Reconstitution of the E. Coli Membrane \(\beta \)-Barrel Assembly Machine from Purified Components

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Abstract

β-barrel membrane proteins perform important functions in the outer membranes of Gram-negative bacteria and in the mitochondria and chloroplasts of eukaryotes. Cellular machines that have been conserved from bacteria to humans assemble these proteins by an unknown mechanism. The components of the β-barrel assembly machine (Bam) in *E. coli* have been identified, but it has been difficult to study their function in vivo because they catalyze an essential process; mutations in proteins involved in the assembly pathway are often lethal or produce pleiotropic phenotypes that do not reveal the specific roles of the individual proteins. This study describes an in vitro reconstitution of the activity of the Bam complex and the use of this assay to determine how the Bam proteins contribute to the assembly of the complex itself.

A sensitive assay for β-barrel assembly was developed using a substrate protein that has protease activity when it is folded. A peptide bond cleavage thereby reports on the conformational change the Bam complex catalyzes. This assay demonstrates that the Bam proteins dramatically increase the rate of β-barrel assembly without any external energy source. The structures of these proteins must inherently facilitate the folding and insertion process.

The in vitro reconstitution was then adapted to study the assembly of the central component of the Bam complex, BamA. These studies reveal that the conserved domains of BamA catalyze the steps in the assembly process that are common in all organisms. The accessory components of the Bam complex adapt the mechanism of BamA to improve its

efficiency and to allow it to handle a diverse set of substrates. The assembly of the Bam complex thus demonstrates how a cellular machine evolves to achieve generality and high efficiency.

A structure of the Bam complex will be required to understand the molecular details of how substrate proteins are bound, folded into β-barrel structures, and inserted into the membrane. Initial efforts indicate that it will be possible to obtain such a structure. By combining structural and biochemical information garnered from the in vitro reconstitution, the general principles that guide the assembly of membrane β-barrels may be determined.

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Chapter 1: Introduction to β-Barrel Membrane Protein Assembly by the Bam Complex

1.1 Protein Assembly in Cellular Membranes

There are only two known classes of integral membrane proteins in all prokaryotes and eukaryotes: α-helical proteins and β-barrel proteins (1)[1]. Because polypeptides consist of amide bonds, which are polar, all membrane proteins must internally satisfy the hydrogen bonds of the peptide backbone. Helical and β-barrel membrane proteins satisfy this requirement in different ways: α-helices make hydrogen bonds between proximal residues while distal residues must form hydrogen bonds in order to close a β-sheet into a cylindrical barrel. Consequently, these proteins must be assembled in different ways. Hydrogen bonds can form sequentially as a polypeptide folds into a helical structure, and provided that the side chains of the amino acids are hydrophobic, the folded helix can be inserted into the membrane. A helical bundle can then form by association of the individually inserted α-helices. In contrast, β-barrels do not have a fully hydrophobic exterior until their tertiary structure is complete. The folding and membrane insertion of β-barrel proteins are therefore likely coupled, but the mechanism remains mysterious.

The cellular machines that assemble α-helical and β-barrel proteins contain components that have been conserved from bacteria to humans. The majority of membrane proteins are α-helical and their assembly has been extensively studied (2, 3). β-barrel proteins are found in the outer membranes of Gram-negative bacteria and in the mitochondria and chloroplasts of

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[1] Much of this chapter is reprinted, with permission, from the Annual Review of Biochemistry, Volume 80.

eukaryotes. This chapter describes the components of the β-barrel assembly pathway and the current understanding of how they function together to facilitate the folding and insertion of these proteins. It will focus on the pathway in *Escherichia coli* and highlight features of this pathway that have been conserved across prokaryotes and in higher eukaryotic systems.

Gram-negative bacteria, including *E. coli*, contain both types of integral membrane proteins segregated between two membranes. These bacteria contain a double membrane cell envelope (4). The inner, cytoplasmic membrane (IM) is a phospholipid bilayer containing integral α-helical proteins and peripheral lipoproteins, which carry out energy driven transport processes. The outer membrane (OM) is an asymmetric bilayer composed of phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet; it is not energized but also contains lipoproteins and integral β-barrel proteins, which create pores in the membrane to allow nutrients and solutes into the cell and waste products out of the cell. The aqueous compartment between these membranes contains the peptidoglycan cell wall and soluble proteins and is termed the periplasm. Since the 1960s when this cellular architecture was first revealed by electron microscopy (5), the biogenesis of the two different membranes has been of interest, and the machines that assemble proteins in these membranes have been identified. The secretion (Sec) machinery assembles α-helical proteins in the inner membrane and is responsible for translocating periplasmic and outer membrane proteins (OMPs) from the cytoplasm (2, 3, 6, 7). The Lol (localization of lipoprotein) machinery transports lipoproteins from the IM to the OM (8), and the Bam (β-barrel assembly machine) complex folds and inserts β-barrels in the OM (9-11). *E. coli* therefore serve as a simple model system in which the assembly of the major classes of membrane proteins can be studied.

1.2 Cytoplasmic Synthesis, Targeting, and Secretion

All integral membrane proteins are synthesized in the cytoplasm with N-terminal signal sequences that direct them to the secretion machinery in the inner membrane (12). However, following their synthesis, α-helical and β-barrel proteins are delivered to the Sec machine by distinct pathways (Figure 1.1). Inner membrane proteins are co-translationally targeted to the Sec machinery. As these proteins are synthesized on the ribosome, a protein-nucleic acid complex, the signal recognition particle (SRP), binds to their N-terminal signal sequences and transports them to the Sec machine. Outer membrane proteins, by contrast, are post-translationally directed to the Sec machine (2, 13). To prevent co-translational secretion of these proteins, an additional protein called trigger factor (TF) competes with the SRP for binding to their signal sequences as they emerge from the ribosome (14-19). The chaperone protein, SecB, then binds to OMPs as they elongate and subsequently directs them to the Sec machine (20, 21). Therefore, although both α-helical and β-barrel membrane proteins are directed to Sec, they are handled differently in the cytoplasm to ensure their proper targeting and assembly.

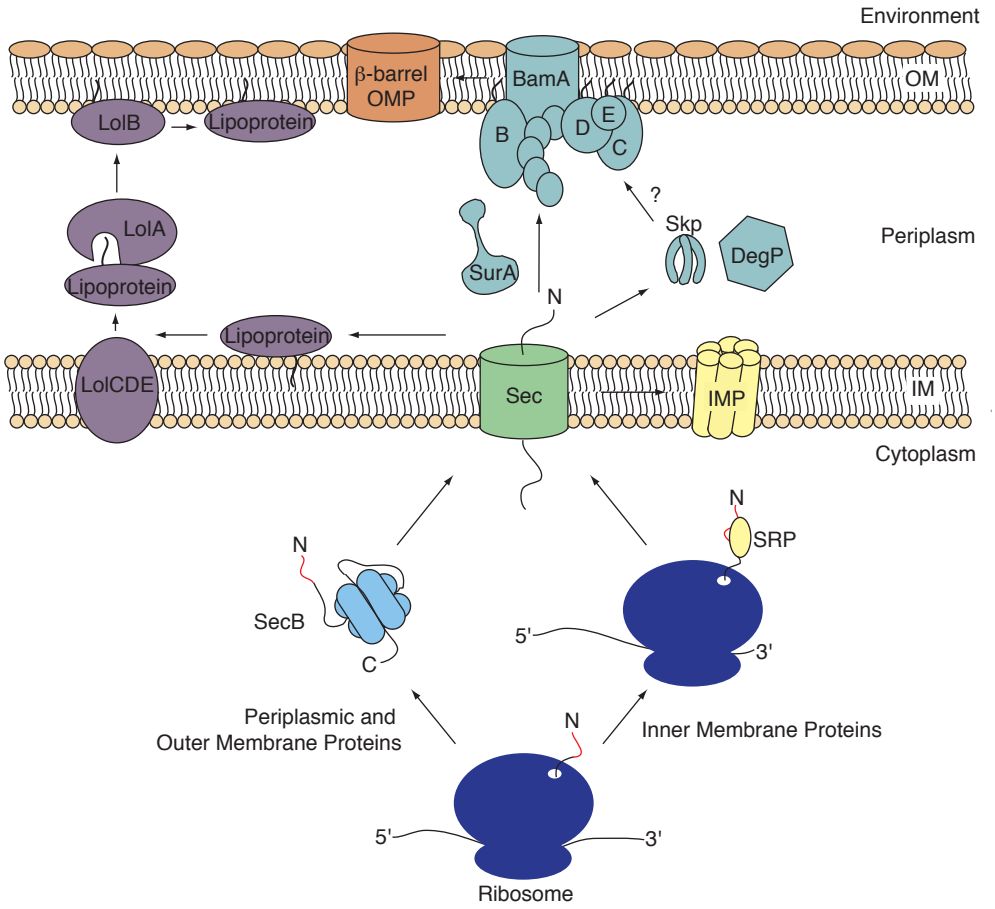

Figure 1.1. Cell envelope protein biogenesis. All proteins destined for the periplasm and two membranes are ribosomally synthesized in the cytoplasm. Inner membrane proteins (IMPs) are then co-translationally directed to Sec and inserted into the membrane, while periplasmic and OM proteins are post-translationally translocated. Soluble, periplasmic proteins then fold in this second aqueous compartment. OM lipoproteins are lipidated at the outer leaflet of the IM and then transported to the OM by the Lol pathway. β-barrel OMPs transit the periplasm in unfolded states with the help of chaperones (primarily SurA) and are then folded and inserted into the OM by the five-protein Bam complex.

Although membrane proteins are more stable in the hydrophobic membrane environment, in all cells, protein complexes facilitate their insertion into membranes. The Sec machine inserts inner membrane proteins using the energy provided by their synthesis on the ribosome. The mechanism by which these proteins are inserted into the membrane has received considerable attention (2, 22). Many of the details still require clarification, but the general model suggests that α-helical segments fold and are individually released laterally into the membrane through a gate in the channel (23). Inner membrane proteins therefore are never exposed to an aqueous

environment during their biogenesis. Outer membrane proteins (OMPs) are translocated through the Sec pore using the energy provided by an ATPase, SecA (24). The post-translational targeting of nascent β-barrel proteins and the lower hydrophobicity of their sequences allow them to be translocated more rapidly, but requires that they be kept in a soluble, folding-competent state (25). These proteins are translocated in an unfolded state and then interact with chaperones in the periplasm in order to prevent them from aggregating or misfolding in this second aqueous compartment. There is no ATP outside the inner membrane; therefore, the proteins responsible for maintaining OMPs in folding-competent states in the periplasm and for assembling them in the OM likely do so without using energy.

1.3 Periplasmic Transport

Proteins with a propensity to form β-sheet structures are also prone to aggregation given the stability of amyloid-like, multimeric structures. To transit the periplasmic compartment in an unfolded state, chaperones are required to bind OMPs after they exit the Sec channel and have their signal sequences cleaved by the signal peptidase (26). The periplasmic chaperone, SurA, has been shown to transport the bulk mass of OMPs to the outer membrane. A parallel pathway that relies on two other periplasmic proteins, Skp and DegP, has been shown to compensate for the absence of SurA and may be more important for handling proteins that have fallen off the efficient assembly pathway (27).

SurA consists of an N-terminal domain, two peptidyl-prolyl domains (P1 and P2), and a C-terminal domain; crystal structures reveal that the N- and C-terminal domains and the P1 domain form a globular core with a crevice that can accommodate extended peptides, while the P2 domain is connected to this domain by 30 Å flexible linkers and demonstrates peptidyl-

proline isomerase (PPIase) activity in vitro (28-31). The core domain contains the chaperone activity of the protein and is structurally similar to the C-terminal domain of trigger factor (15, 30). This striking structural similarity may reflect the fact that SurA and TF have both evolved to recognize features of unfolded proteins and prevent them from misfolding in their respective compartments (32).

SurA has been shown to bind to model peptides and unfolded OMPs with low micromolar affinity and to prefer substrates rich in aromatic residues arranged in alternating sequences (Ar-X-Ar) (33-35). These sequences are much more commonly found in OMPs than in soluble or inner membrane proteins (33, 35). Strains lacking SurA exhibit defective outer membrane phenotypes and have decreased levels of the major OMPs including OmpA, LamB, OmpC, and OmpF (27, 29, 36, 37). Kinetic analysis of the assembly of LamB indicated that SurA is important in the transformation of unfolded LamB monomers to folded monomers and affects the rate of signal sequence cleavage, suggesting that SurA may interact with unfolded OMPs while they are translocating across the inner membrane (38). While the bulk mass of OMPs are transported by SurA, it is not an essential protein and most OMPs can utilize other chaperones (37).

The minor periplasmic chaperone, FkpA, also possesses separable PPIase and chaperone activities, but unlike SurA, genetic inactivation of this protein does not produce observable defects (39-42). Deletion of FkpA and members of the Bam complex do produce synthetic phenotypes, and a strain containing a quadruple deletion of SurA, FkpA, and two other periplasmic PPIases, PpiA and PpiD, exhibits diminished growth and increased antibiotic sensitivity relative to the single deletions (42, 43). FkpA thus clearly plays a supporting role under normal growth conditions but may be more important under stress conditions when

efficient OMP assembly is critical. Therefore, it may be useful to understand the role of this protein in relation to the major OMP chaperones and whether it can deliver unfolded substrates to the assembly machinery in the outer membrane.

Skp has received considerable attention as an OMP chaperone, but its precise role is also not clear and several hypotheses about its function have been generated. Skp (seventeen kilodalton protein) is a trimer in its functional form and has been shown to bind denatured OMPs (44, 45). Each Skp monomer contributes an α-helical "tentacle" domain to the "jellyfish" trimeric structure to produce a large, hydrophobic cavity that can accommodate molten globule states of OMPs (46). Recent studies have demonstrated that the β-barrel domain of OmpA is bound in an unfolded state within the Skp cavity, while the periplasmic domain of OmpA remains outside the cavity and can fold independently (47, 48). The fact that it can be cross-linked to the Sec machine in vivo has led to the hypothesis that Skp acts early in the biogenesis of OMPs and then hands them to SurA for delivery to the OM (49, 50). In accord with this hypothesis, a β-barrel protein, EspP, has been shown to cross-link to Skp at early time points in its biogenesis and to SurA at later time points when EspP also interacts with the Bam complex (51). However, only a small percentage of the EspP observed in this study was cross-linked to the chaperones, so it is not clear whether these species represent intermediates in a single pathway. A direct interaction between Skp and SurA has also never been observed, and this model of their sequential involvement is difficult to reconcile with the genetic evidence that these chaperones function in separate, parallel pathways. Either protein can be deleted individually, but the double deletion is lethal (27).

Conversely, others have suggested that Skp may act late in the assembly pathway because it contains a putative LPS binding site, and LPS, in concert with Skp, has been shown to improve

the efficiency of OmpA assembly into lipid bilayers (44, 46). OmpA folding was also shown to depend on pH and the negative charge of lipids in the membrane (52). It is difficult to evaluate whether the LPS-facilitated assembly in this in vitro system is relevant in the in vivo system where the Bam complex participates in OMP assembly. However, the possible involvement of LPS in Skp function may suggest that Skp could be more important under conditions when the cell experiences stress. Such stress might also result in defects in LPS biogenesis that would allow LPS to interact with Skp; under normal conditions, LPS is only found in the outer leaflet of the OM and thus would not be expected to encounter Skp (53). The possibility that Skp primarily sequesters substrates that have fallen off the more efficient SurA folding pathway is attractive given that Skp is believed to function in the same pathway as the OMP degrading machine, DegP (27).

The accumulation of misfolded or aggregated proteins in the periplasm would be toxic; therefore, cells have developed intricate pathways for recognizing the presence of such species and activating stress responses, which produce proteins that sequester and degrade the misfolded proteins. All of the periplasmic chaperones and the Bam complex in the OM are regulated by σ^E cell envelope stress response (43, 54-57). The expression of the periplasmic protease, DegP, is tightly controlled by the σ^E and Cpx regulons (58), and its activity is further regulated by the oligomeric state of the protein. The resting state of the protein is hexameric, but upon binding an unfolded OMP, it converts to large cage-like structures containing 12 or 24 monomers (59-62). The protease is inhibited in the hexameric state by a loop in a PDZ domain from a neighboring monomer; in the 12- and 24-mer structures, this loop is displaced from the active site. The active cages remain assembled until the substrate is fully degraded and then rapidly disassemble (62).

This assembly and disassembly mechanism ensures that the protease is only active when unfolded proteins need to be degraded.

The careful control of the assembly of the DegP proteolytic cages seems inconsistent with a chaperone role for these structures. However, a cryo-EM structure of the 12-mer revealed a folded OMP monomer contained within the cage; this observation and the finding that the 24-mer associates with lipid membranes has lead to the hypothesis that DegP acts as a chaperone that may be able to directly insert folded OMPs into the outer membrane (60). These observations may be artifactual; the folded OMP could have been trapped within the 12-mer during the protein purification process. Nevertheless, if these DegP-OMP complexes are physiologically relevant, they are likely part of a minor assembly pathway. DegP, unlike the Bam complex, is not essential for viability.

The cell has evolved similar proteins to handle unfolded and misfolded proteins in the cytoplasm and periplasm. SurA is structurally similar to trigger factor, and the large cage-like DegP structures are reminiscent of the cytoplasmic GroEL chaperone and proteasome degradation machineries (15, 61). Clearly, there are conserved mechanisms for maintaining proteins in folding competent states and for removing those that fall off the folding pathway, but the details of how unfolded OMPs in these two pathways are managed still need to be clarified. Chapter 2 will describe an assay that demonstrates that SurA can deliver unfolded substrates to the Bam complex in vitro (63). This assay could be used to understand the molecular basis of SurA function; the stoichiometry of the binding of SurA to its substrates could reveal how it maintains their folding competent state and whether it actively participates in the folding process. It also remains unclear how Skp and DegP function together to handle proteins that fall off the efficient folding pathway and whether those proteins can reenter the folding pathway. Finally, it

will be interesting to determine whether the features of the unfolded substrate that are recognized by the chaperones are also important for recognition by the Bam machinery.

1.4 Outer Membrane Recognition and Assembly

The assembly steps that occur once OMPs have reached the OM are the least well understood, but in the past 10 years, significant progress in this area has been made as the multi-protein complex responsible for facilitating these steps has been identified. Studies of the structure and function of this complex are beginning to address the fundamental questions of how unfolded OMPs are recognized as β-barrels at the OM and how they are assembled into their folded structures and inserted into the membrane.

1.4.1 Identification of the Assembly Machine in the Outer Membrane

Identifying the factors involved in OMP assembly at the OM had been very difficult. Before a large number of bacterial genome sequences were available, OMPs could only be identified by fractionation—that is, by separating the outer membrane from other cellular components and isolating the constituent proteins. This classic approach was able to identify a few highly abundant outer membrane proteins, including Braun's lipoprotein, OmpA, OmpC, and OmpF (64-66), but not the low abundance proteins, which are responsible for assembling the OM.

It has long been assumed that the machinery for assembling proteins in the outer membrane must be essential, since the outer membrane is essential. However, when work began on the identification and characterization of the Bam assembly machinery, only two outer membrane proteins had been shown to be essential, LptD (formerly Imp) and LolB (67-69).

LolB is an outer membrane lipoprotein that interacts with a periplasmic chaperone, LolA, which delivers newly synthesized lipoproteins to the outer membrane. LolB thus determines where newly synthesized lipoproteins are inserted. LptD is an integral outer membrane protein identified in genetic selections for altered OM permeability. LptD was the first essential OM β-barrel protein discovered in *E. coli*, and depletion analysis of LptD indicates that it plays a role in LPS assembly (68, 70, 71). Certain *E. coli lptD* alleles that encode proteins containing deletions compromise the function of LptD, leading to increased membrane permeability and giving LptD its former name (Imp) (67). The knowledge of these two proteins implied that there should be essential machinery in the OM for assembling integral OMPs, but additional techniques were still required to discover the components of that machine.

The availability of large numbers of bacterial genome sequences enabled the discovery of the central component of the assembly machine, BamA (or originally Omp85), in *Neisseria meningitidis* by searching for sequence conservation and similarity to the chloroplast homolog (72, 73). This protein was then clearly shown to be involved in β-barrel assembly (73). However, the other members of the Bam complex are less well conserved and not all are essential, so it was not possible to identify them from genome sequences. Traditional genetic selections were also not successful in identifying these proteins because the cell typically responds to gross defects in OM permeability by producing mutations in many different genes, which were difficult to deconvolute.

This problem was overcome by a different approach in which specific chemical conditions provided a specific selection pressure to which the cell responded with specific mutations. This "chemical conditionality" approach has its origins in Pardee, Jacob, and Monod's seminal studies on the *lac* operon (74, 75). They used galactoside analogs to

investigate the regulation of gene expression, but the general principles can be expanded to large numbers of different chemicals, which challenge cells in a multitude of different, specialized ways. Because membrane permeability is dictated by membrane composition and structure, this approach potentially could be used to identify factors involved in any aspect of making a membrane, from the beginning (synthesis of components) to the end (assembly) and any of the intermediate steps (targeting).

Specifically, a chemical genetic screen in an *E. coli* strain with a leaky outer membrane identified the second component of the assembly complex, BamB (formerly YfgL) (76). The screen made use of a strain that contains a mutant *lptD* allele, *lptD4213* (formerly *imp4213*), that confers OM permeability defects; this strain was treated with a set of molecules that are toxic to cells if they can reach their targets in the periplasm. The selection produced suppressor mutations in BamB that specifically decrease the permeability of the OM to the toxic molecules. It is remarkable that these mutations restore the barrier function of the OM such that the toxic molecule used in the selection is excluded but other toxic molecules are not (77, 78). Different small molecules select for mutations in different components of the machinery involved in membrane biogenesis presumably because each component contributes differently to membrane integrity and the small molecules reveal those differences. The cell thus tunes the OM permeability to the specific chemical condition applied and thereby reveals more subtle effects of OMP assembly factors.

Although the chemical genetic screen identified BamB, it was not trivial to determine its function. Although it was simple to determine that it is an OM lipoprotein, it is not an essential protein and there were no known homologs. Affinity chromatography experiments demonstrated that it stably associates with BamA (formerly YaeT in *E. coli*) and three other lipoproteins

BamC, D, and E (formerly NlpB, YfiO, and SmpA, respectively) and is thus part of an important assembly complex (77, 79, 80). The interactions between the complex components are stable and specific; any member of the complex can be His-tagged and used to pull down all of the other components. Moreover, the isolated complex runs as a single band on blue native gel electrophoresis—a more stringent measure of the stability of the interaction of these proteins (63, 81). More recently, Omp85 in *N. meningitidis* was also been shown to be part of a complex containing homologs of BamC, D, and E and a fourth protein, RmpM (82). It seems increasingly clear that many of the components of the complex are found across bacterial species (83-85).

Two of the components of the complex, BamA and BamD, are essential (79, 86). BamA is a predicted integral β-barrel protein with a large periplasmic domain and is discussed in detail in the next section. BamD is an α-helical OM lipoprotein that contains five tetratricopeptide repeats (TPR). TPRs commonly mediate protein-protein interactions and therefore could be important in interactions among the complex members or with substrate proteins (84). The first three TPRs of BamD form a groove which has been proposed to bind the C-terminus of OMP substrates, and the remaining two TPRs contain sequences that, when mutated, affect the binding of other Bam components (81, 86-89). OMP assembly is undoubtedly a multi-step process that involves binding unfolded substrates, folding them, and inserting them into the membrane. It is an interesting question whether the two essential proteins of the Bam complex function in concert throughout the process or are responsible for completing separate steps.

BamB, BamC, and BamE are also lipoproteins and are not essential. However, deletions of any of these lipoproteins also produce defects in OMP biogenesis as indicated by lower levels of folded OMPs in the OM, induction of the σ^E stress response, and increased sensitivity to bile salts and antibiotics (43, 79, 80, 86). Deletions of BamB and other proteins in the OMP

biogenesis pathway (including the chaperones SurA, DegP, and FkpA) produce synthetic phenotypes and indicate that it may play a role in determining the efficiency of assembly (43, 90, 91). It has been suggested that the β-propellar structure of BamB could present multiple binding sites for the β-strands of substrate OMPs and thereby aid in assembling proteins or in delivering them to BamA (92-94). BamC and E are important for the stability of the complex but are less well conserved among bacterial species, and deletions of these proteins produce less severe phenotypes (80, 84). However, BamC and BamE do appear to have distinct functions. Deletions of BamE and other components of the OMP biogenesis pathway produce more severe synthetic phenotypes than the corresponding double deletions with BamC. BamA is also rendered more sensitive to protease in the absence of BamE, which may suggest that BamE plays a more important role in affecting the stability of BamA (95). Unfortunately, the structures of BamC and BamE have not substantially clarified how these proteins perform their functions (88, 89, 96-98). The essentiality of BamA and BamD implies that they are primarily responsible for the performing the chemistry required to fold and insert OMPs. However, by understanding how BamB, C, and E modulate the activity of the essential proteins, we may learn how the Bam complex overcomes the energetic barriers to OMP assembly or how its activity is coordinated with other cellular processes.

1.4.2 Structure and Function of BamA

BamA, the central component of the assembly complex, is conserved across all Gram-negative species, and there are orthologs of it in the mitochondria and chloroplasts of eukaryotes. All of the proteins in this superfamily contain one or more soluble polypeptide transport associated (POTRA) domains. All BamA orthologs in Gram-negative bacteria contain five

POTRA domains, while those in chloroplasts and mitochondria contain three and one, respectively (72, 99-101). In bacteria, these POTRA domains are found in the periplasm and receive substrates translocated across the inner membrane. The mitochondrial β-barrel proteins are synthesized outside the mitochondria (in the cytoplasm) but are not inserted directly in the mitochondrial OM from the outside. Instead they are translocated into the intermembrane space and then assembled by the BamA ortholog called Sam50 (or Tob55) from the inside. The fact that assembly in mitochondria occurs from the same face of the membrane as in bacteria suggests that the process of β-barrel assembly has retained features of the prokaryotic systems from which these organelles originated (11, 100, 102, 103). Given that the structure and function of this protein has been conserved from bacteria to humans, studies of how BamA assembles OMPs could reveal general principles that all cells follow in assembling integral membrane β-barrels.

The POTRA domains of BamA have been the subject of crystallographic and NMR structural studies that have shed light on how the domains scaffold the lipoprotein components of the complex, on the importance of certain POTRA domains in the function of the complex, and on how unfolded substrate polypeptides might interact with these domains (81, 104-106). Each domain consists of approximately 75 residues arranged in two anti-parallel α-helices folded on top of a three-stranded β-sheet. The domains have low sequence but high structural similarity; the conserved residues are found primarily in the hydrophobic core of each domain (81, 107). The structures show that the domains are modular, suggesting that each domain can fold independently; they are connected by linkers and do not appear to make significant contacts with each other. The structures also defined where each domain starts and ends, which made it possible to construct individual deletions of the domains.

The POTRA domains are responsible for binding the four lipoprotein components of the complex. Pull-downs using the POTRA deletion constructs indicate that BamC, D, and E bind to POTRA domain 5 (P5) and that the association of BamB is affected by the deletion of any of the P2-5 domains (81). Therefore, BamB associates with BamA separate from BamC, D, and E. This observation was used to develop methods to over-express and purify large quantities of the five-protein complex, as described in Chapter 2 (63). Briefly, BamA and B were over-expressed in one strain and BamC, D, and E in a second, and the five-protein Bam complex was then reconstructed in vitro (63). The fact that the two essential proteins, BamA and D, can be expressed separately without interfering with the function of the native, complete Bam complex may indicate that these proteins have separate functions, which must be coordinated.

POTRA domains 3-5 are essential in *E. coli* (81). It is not surprising that P5 is essential because it scaffolds the essential lipoprotein, BamD; it is thus required to assemble a functional Bam machine. The fact that P3 and P4 are essential but do not scaffold an essential component of the machine implies that they play a direct role in the assembly of OMP substrates. The P5 deletion produces an interesting phenotype even in cells simultaneously expressing wild-type BamA—namely, it is toxic (81). Therefore, BamA lacking P5 can compete with wild-type BamA in these cells in some way that directs OMPs off pathway. One possible explanation for this dominant negative phenotype is that the deletion allows substrates to start, but not complete folding. In this model, the essential function of BamD would occur after the initiation of OMP assembly on BamA.

Since the Bam complex assembles all OMPs, it must assemble BamA as well. Some of the POTRA domains are clearly required for proper BamA assembly. Remarkably, in the absence of full length BamA, BamAΔP2 is able to assemble itself in the OM and exhibits growth

like the wild-type control (81). BamAΔP3 and ΔP4 were stably expressed in the presence of the full length copy, but were not detected by Western blot analysis in its absence, suggesting that these variants could not properly assemble themselves and were consequently degraded (81). This observation is consistent with the essentiality of these domains, but they may have additional essential functions. The deletion of P1 had more moderate effects on viability and OMP levels, and a recent study on the effects of smaller deletions in P1 suggested that this domain also affects the assembly of BamA (108). An interesting question is whether the POTRA domains participate intramolecularly in the assembly of the BamA β-barrel and thereby function as a self-chaperone or whether they affect the assembly of other BamA molecules once they are assembled in the OM. Chapter 3 describes in vitro studies of the assembly of BamA by the Bam complex that help to clarify some of these points.

The essentiality of the POTRA domains in other organisms is still an open question. In mitochondria, the BamA ortholog contains only one POTRA domain, which appears to be essential for release of OMPs from the assembly machine (109-111). In the *N. meningitidis* ortholog, sequential truncations of the POTRA domains indicated that only P5 is essential for viability in this organism (112). Interestingly, LPS is not essential in *N. meningitidis* and this organism also lacks a homolog of BamB (82). The fact that suppressor mutations in BamB were the response to a defect in the machinery that assembles LPS suggests that BamB may help coordinate LPS assembly or the assembly of LptD, which is responsible for LPS assembly (76, 77, 79). Furthermore, it has been suggested that SurA chaperones LptD and that the functions of SurA and BamB are related (37, 38, 43, 77). Thus, the differences in the essentiality of the POTRA domains may reflect the different OM assembly requirements in these two bacterial species. The fact that the number of POTRA domains found in the mitochondrial, chloroplastic,

and bacterial orthologs differ but have been conserved within their respective kingdoms must also reflect the different requirements of the membranes in which these assembly machines exist.

Structures of the POTRA domains have provided evidence for a possible mechanism by which they could interact with unfolded OMP substrates and facilitate OMP assembly and which may explain the essentiality of P3 (Figure 1.2). Two crystal structures of a construct containing P1-4 and a fragment of P5 were independently determined; in both structures, the fragment of P5 bound to the β2-strand of P3 in a second monomer (81, 104). The fragment extended the β-sheet of P3 by an additional strand—a binding mechanism that has been termed β-strand augmentation (113). The additional strand bound in a parallel orientation in one structure (81) and antiparallel in the other (104). An NMR study has also shown that OMP-derived peptides can interact with the β1-strand of P1 and the β2-strand of P2 and that an α-helical peptide with a sequence derived from the periplasmic maltose binding protein does not interact (105). This binding mechanism appears to be specific for β-strand secondary structure rather than a particular amino acid sequence and therefore is an attractive mechanism by which OMP substrates could bind to and be recognized by BamA. In fact, the machinery required to sense misfolded OMPs in the periplasm also binds β-strands by β-augmentation (114-116). Since the Bam complex handles a large number of different substrates, it is an interesting question whether it recognizes its substrates through a general motif—like propensity to form β-structure—or uses a specific signal sequence.

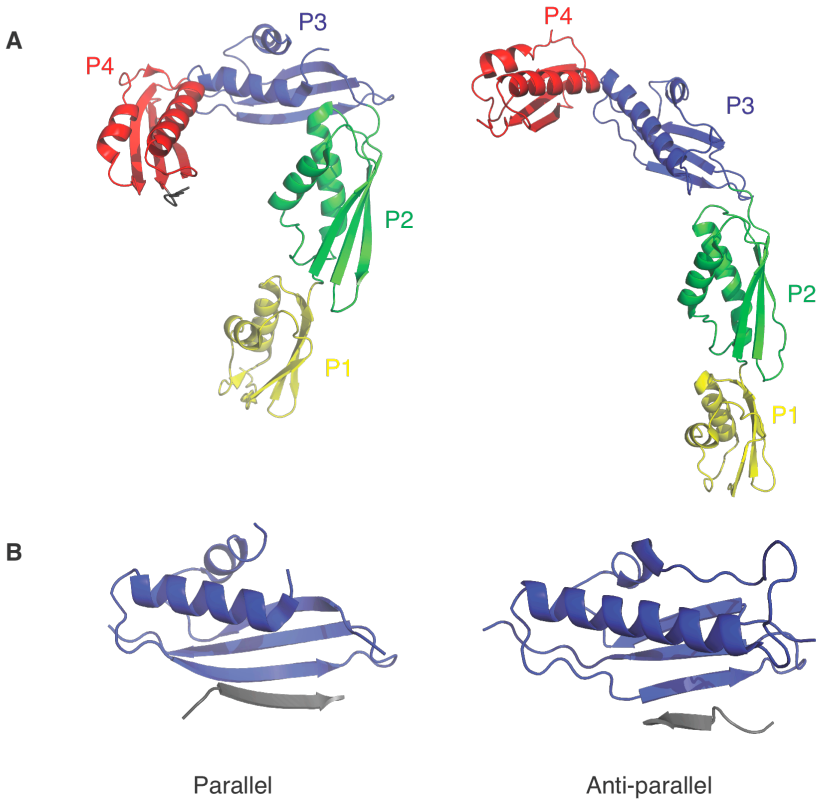

Figure 1.2. Crystal structures of the POTRA domains of BamA. **A.** Very similar fragments of BamA containing the first four POTRA domains and a short peptide from P5 crystallized in different conformations. The fishhook (PDB: 2qdf) and more extended (PDB: 3efc) structures suggest that there may be a flexible hinge between P2 and P3, which could help to generate β-hairpins in the OMP substrates of BamA. **B.** β-strand augmentation interactions observed in both crystal structures. The POTRA domains crystallized as a dimer in both structures in which the short peptide from P5 (gray) bound to the β2-strand of P3 (blue) of a second monomer extending the β-sheet by an additional strand. P3 bound the peptide in parallel (left) and anti-parallel (right) orientations.

The two POTRA domain crystal structures demonstrate that the β-augmentation can occur through a parallel or anti-parallel interaction. If the hypothesis is correct that BamA binds incoming OMP substrates by this mechanism, the two orientations may suggest that during the assembly of an OMP substrate, the POTRA domains bind segments of the OMP in alternating orientations. Furthermore, the two different conformations of the POTRA domains in the determined crystal structures—one fishhook and one more linear—indicate that there is flexibility between P2 and P3 (81, 104). Whether this "hinge" is functionally relevant remains to

be determined, but if the POTRA domains actively participate in assembling substrates, we would expect them to move during that process. The β-augmentation may initially facilitate the formation of β-structure in the substrate, but the β-strands would still need to be assembled into β-hairpins and ultimately into a closed barrel. This tertiary structure formation would seem to require conformational changes in the assembly machine in order to facilitate the formation of hydrogen bonds between distal residues along the two edges of the β-sheet that come together to close the β-barrel.

The POTRA domains are in the periplasm and may be able to start the assembly process, but the OMP substrates must ultimately be inserted into the membrane. It seems reasonable to assume that the β-barrel domain of BamA plays a role in these later steps in the membrane. Chapter 4 describes efforts to crystallize a Bam subcomplex containing the BamA β-barrel, but a structure has not yet been determined. Current hypotheses about the structure and functional role of the BamA β-barrel rely on bioinformatic predictions, which suggest that it contains 12 or, more likely, 16 β-strands, and on comparisons to homologous proteins (101, 117). The structure of FhaC, a member of the Omp85-TpsB superfamily, has been determined (118). FhaC is a component of the two-partner secretion (TPS) system that secretes the β-helical protein, filamentous hemagglutinin (FHA), in *Bordetella pertussis*. It contains two POTRA domains that are required for secretion of FHA and a 16-stranded β-barrel. A long loop (L6) between strands 11 and 12 of the barrel is folded back inside the barrel extending to its periplasmic face; this loop is required for secretion and is believed to undergo conformational changes that expose it to the extracellular environment during secretion (118, 119). The current model of FhaC-mediated secretion involves recognition of the unfolded FHA by the POTRA domains, which direct the protein into the β-barrel where it interacts with loop L6; this interaction causes L6 to flip out of

the barrel dragging the FHA protein with it. BamA is also predicted to contain a long loop between two conserved regions of the β-barrel (117, 120), and mutations that suppress the OMP assembly defects associated with a double deletion of BamB and BamE map to this predicted loop (121). BamA does not secrete proteins across the membrane, but clearly it would be useful to understand the mechanistic role of this region of BamA.

1.4.3 Mechanistic Studies of the Bam Complex

It seems reasonable to assume that β-barrel assembly is a multi-step process. In order to understand the mechanism of OMP assembly, it is necessary to break the process into steps and determine how the Bam complex facilitates each step. Therefore, it would be very useful to observe intermediates in the process of binding to the Bam complex, folding, and inserting into the membrane. It is challenging to detect such intermediates or to disrupt the process and observe partially assembled OMPs because assembly is a highly efficient and essential process. Furthermore, mutations in the proteins in the assembly pathway often result in pleiotropic phenotypes characteristic of defective OMs (e.g. lower levels of OMPs in the OM, sensitivity to antibiotics and bile salts, induction of the σ^E stress response), but the specific effects of the mutations on the recognition, folding, or insertion of OMPs have been difficult to identify. Nevertheless, several in vivo and in vitro studies have begun to elucidate aspects of the assembly mechanism; not surprisingly, these studies have provided more information about the early steps of substrate recognition and binding to the Bam complex than the later folding and insertion steps, which are inherently more difficult to understand.

1.4.3.1 Substrate Recognition

The Bam complex must distinguish between its substrates and soluble proteins in the periplasm. Proteins are directed to the inner membrane Sec machine by an N-terminal signal sequence, and studies have addressed whether OMPs are directed to Bam by an analogous mechanism in which features of the substrate's sequence determine its targeting. Those features could relate to the substrate's ability to form β-strands with hydrophobic periodicity (via β-augmentation), could be a specific "signal" sequence of amino acids, or could be combined with features of the periplasmic chaperones that deliver OMPs.

It has been suggested that a specific amino acid sequence at the C-terminus of β-barrels could be important for recognition by BamA (122, 123). BamA was reconstituted into planar lipid bilayers and shown to exhibit channel activity that could be altered by the addition of denatured PhoE or LamB. Deletion of the C-terminal phenylalanine of PhoE abolished the channel opening activity, and peptides containing the last 12 or 11 residues of PhoE mimicked the activity of the full-length and mutant protein, respectively (123). Similarly, the Sam complex exhibited channel activity in planar lipid bilayers, and that activity was altered by the addition of a peptide containing a sequence from the C-terminal strand of mitochondrial OMPs (110). The structure of BamD revealed a pocket formed by the first three TPRs that is similar to pockets in other proteins that bind C-terminal peptides; this observation might lend support to a C-terminal recognition mechanism, but it is not obvious how it explains the channel activity experiments, which were performed in the absence of BamD (87-89).

The structure of the C-terminus of β-barrels is undoubtedly important in their assembly and stability since it is required to close the barrel. In fact, many β-strands in a β-barrel end in aromatic residues such that the assembled barrel contains aromatic "girdles" at the membrane

surfaces (124, 125). The channel activity of BamA is clearly affected by aromatic residues, but whether that activity correlates with OMP assembly is unclear. Since proteins are translocated across the inner membrane from N- to C-terminus, OMPs would need to be completely translocated across the inner membrane before the Bam complex could recognize them. In that regard, it is worth noting that the five POTRA domains on BamA are of sufficient length to span a significant portion of the periplasm and thus might interact with substrates as they emerge from the Sec machine (104). It remains an open question whether translocation could be coupled to assembly in the OM, but the answer would have clear implications for how OMP substrates are first recognized by the Bam complex.

Chaperones facilitate the transit of substrates across the periplasm, but it is not clear whether they interact directly with the Bam complex or simply maintain substrates in a folding-competent state. SurA has been shown to cross-link to the Bam complex in vivo, and cross-linking to BamA does not appear to depend on the presence of BamB (27, 90). Similar amounts of SurA were cross-linked to BamA in the presence or absence of BamB, and a region in the first POTRA domain of BamA near residue R64 was specifically shown to cross-link to SurA (90, 108). Interestingly, Skp has never been shown to cross-link to the Bam complex in vivo—consistent with its less central role in the assembly process (27). These studies suggest that SurA can be in close proximity to BamA but do not reveal whether there is specific recognition of the chaperone or if the chaperone is associated with the Bam complex during folding of a substrate and influences the folding process.

Genetic interactions and kinetic studies, however, support the idea that SurA is involved in the assembly steps that occur at the outer membrane. The synthetic, severely defective phenotype of a strain lacking both SurA and BamB suggests that these proteins play related

functions (43, 77). Individual deletions of these proteins also produce identical defects in the kinetics of assembly of LamB—specifically affecting the transformation of unfolded monomers to folded monomers (38). These studies implicate BamB in the initial steps of OMP assembly at the OM; BamB may not be required to recognize and bind SurA-bound substrates (as it is not an essential protein and is not required for cross-linking), but may facilitate their interaction with BamA. Chapter 2 describes an in vitro reconstitution of OMP assembly that indicates that BamB is required for efficient assembly of an OMP delivered by SurA (63). Therefore, OMPs can interact with BamA and be assembled in the absence of BamB, but this protein dramatically improves the efficiency of the process.

Several pieces of evidence suggest that multiple SurA molecules aid in folding substrates on the Bam complex. A crystal structure shows that SurA can bind a model peptide as a dimer, and it has been pointed out that OMPs contain multiple sites for SurA binding (126, 127). The involvement of multiple SurA molecules in OMP assembly evokes an attractive model in which sequential dissociation of SurA molecules allows parts of the substrate to begin folding while others remain protected. Consistent with this hypothesis, an autotransporter, EspP, containing a mutation that stalls the secretion of its passenger domain was shown to cross-link to the Bam complex and to the chaperones SurA and Skp; residues in the β-barrel domain cross-linked to BamA while residues in the passenger domain cross-linked to the chaperones (51, 128). This data suggests that this "autotransporter" is actually assembled by the β-barrel biogenesis pathway and that assembly is a sequential process throughout which the substrate is protected by the Bam complex and chaperones.

1.4.3.2 Substrate Folding

Although most OMPs can fold spontaneously in vitro, different OMPs fold with varying degrees of efficiency depending on the pH, temperature, and lipids used in these systems (129). The Bam complex facilitates the assembly of all OMPs and therefore must overcome these intrinsic differences. As previously discussed, the problem of β-barrel assembly suggests that folding and insertion likely occur in a concerted process. Consequently, it is incredibly challenging to observe and interpret the structures of substrates at different stages. Clearly, the work that has revealed the most about partially assembled states has been that of the assembly of a β-barrel in isolated mitochondria. Using a radiolabeled substrate, Tom40, and blue native gel electrophoresis, it was shown that the substrate first associates with the Sam complex, is then inserted into the membrane, and finally assembles into the multi-protein complex in which it functions. N-terminally truncated variants of Tom40 associate with Sam but are not subsequently inserted and assembled in the membrane, and deletions in the last strand of the β-barrel impaired the formation of the Sam-bound intermediate (110). More recent studies have indicated that other components of the Tom complex (into which Tom40 ultimately assembles) are associated with Tom40 while it is still on Sam (130, 131). Therefore, these studies are establishing that intermediates exist but also that the insertion and assembly steps are not entirely distinct.

The in vitro reconstitution of the activity of the *E. coli* Bam complex may make it possible to identify intermediates like those observed in the mitochondrial system and to dissect the roles of the different Bam proteins in the different steps of assembly. Chapter 2 describes the development of this in vitro assay and Chapter 3 describes its application to study the assembly of Bam complex itself. These experiments reveal the minimal requirements for catalyzing β-

barrel assembly and may explain the relative importance of the different components of the Bam components.

1.4.3.3 Substrate Insertion

β-barrels are intrinsically different from α-helical proteins, but an interesting question is whether there are any general principles that guide the assembly of both classes of proteins. Our understanding of the last step of the assembly of β-barrels, membrane insertion, is perhaps the weakest. It is not clear whether insertion could occur through the β-barrel of a BamA monomer or within a hydrophobic cavity created either by the lipoproteins or by the association of multiple Bam complexes. This question has been similarly debated for the Sec machine (23, 24, 132-135). There is evidence that BamA alone forms aggregates; BamA, over-expressed and refolded from inclusion bodies, was shown to form oligomers (up to tetramers) in vitro by size exclusion chromatography and blue native electrophoresis. It was consequently suggested that OMPs could be assembled between the BamA monomers and then released into the membrane by dissociation of the monomers (123). This model provides a proteinaceous environment in which to fold OMPs and a mechanism for insertion that does not require breaking any bonds within the BamA β-barrel. However, it is not clear whether BamA assembles into oligomeric structures in vivo; when the five-component Bam complex is isolated from cells, its apparent molecular weight on a blue native gel is consistent with a monomeric complex (63, 81). Size exclusion chromatography-light scattering analysis of the over-expressed and purified five-protein complex also indicated that it contains only one copy of BamA (63). The oligomerization of BamA in vitro could be an artifact of the lack of the associated lipoproteins, or perhaps the five-protein complex could oligomerize in vivo.

If the complex functions as a monomer in vivo, insertion would presumably be coordinated by the lipoproteins and occur adjacent to the BamA β-barrel or would require local, transient opening of the BamA β-barrel to allow lateral diffusion of substrates into the membrane in a manner analogous to the Sec machine. Breaking hydrogen bonds between strands of the β-barrel within the membrane to allow lateral diffusion from the lumen of the barrel would at first seem impossible. However, other OMPs that handle substrates that must diffuse in and out of the membrane contain regions in which the β-strands are not completely hydrogen bonded. PagP modifies LPS molecules in the OM and FadL imports fatty acids from the environment. Both proteins have crenellated β-barrels in which two adjacent strands are not completely hydrogen bonded near the extracellular edge. The substrates are proposed to pass through this lateral opening in and out of the membrane (136-138). In fact, in vitro thermal denaturation experiments have suggested that the BamA β-barrel is significantly less stable than other OMPs, which may be consistent with an incomplete barrel structure (129). It is more difficult to imagine how an OMP protein could be released through an opening in the BamA β-barrel, but this possibility cannot be ruled out and illustrates the importance of obtaining structural information about this region of the protein.

1.5 A Model of β-Barrel Assembly

The Bam complex facilitates OMP assembly by providing a pathway to folding with a lower energy barrier. The two essential proteins in the complex must be responsible for the chemistry that occurs and the three non-essential proteins presumably modulate their activity. The barriers to OMP assembly include removing the chaperones which solvate the unfolded protein in the periplasm, forming β-hairpins and ultimately bringing the two termini together into

a closed conformation, and rearranging lipids in the membrane in order to insert the folded protein. BamB appears to facilitate the interaction of substrates with BamA perhaps by inducing a more favorable conformation in BamA for SurA to bind (38, 43, 63). The cell can survive without BamB, but OMP assembly is clearly less efficient. β-strand augmentation could then explain how the Bam complex lowers the barrier to structure formation (81, 104, 105). By binding segments of the unfolded substrate the POTRA domains of BamA template the formation of β-strands, thereby paying part of the entropic cost of forming β-structure. If multiple POTRA domains are able to bind β-strands and move relative to one another (as has been suggested) (104), they might be able to bring consecutive strands together to form β-hairpins or bind the first and last strands of the barrel and thereby satisfy the exposed edges of the β-sheet until it is closed into a cylinder and inserted (Figure 1.3).

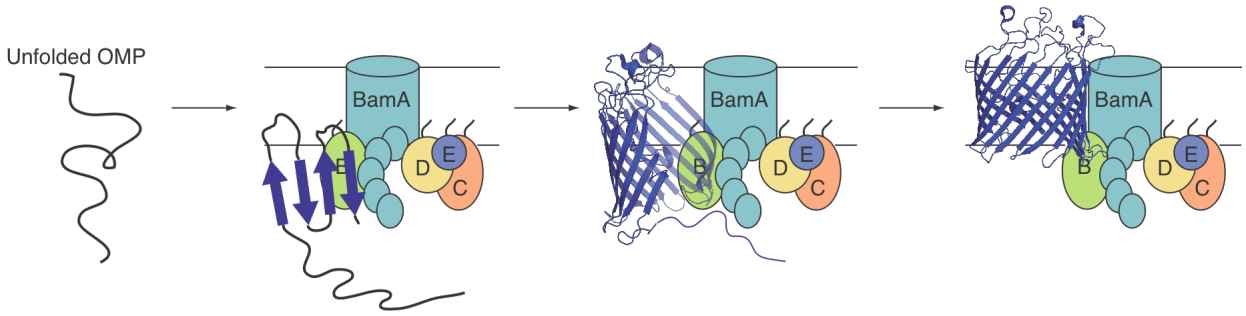

Figure 1.3. A model of how β-strand augmentation could template β-barrel folding. Binding of an unfolded OMP to the POTRA domains of BamA by β-strand augmentation initiates β-structure formation. The POTRA domain thereby satisfies the hydrogen bonds on one edge of the β-sheet as folding proceeds. Ultimately, the two edges of the sheet are brought together and the barrel is inserted into the membrane.

Very little is known about the essential function of BamD. It could be involved in recognizing the C-terminus of OMP substrates, or if BamA is responsible for the early steps in binding and assembling substrates, BamD might be responsible for the later steps of insertion into the membrane and possibly assembly of oligomeric OMPs into their final states. Perhaps BamD helps to dissociate a folded OMP from BamA and release it into the membrane so that the

substrate ultimately satisfies all of its hydrogen bonds internally rather than continuing to make use of those in BamA. Or perhaps the essentiality of BamD reflects a specific role it plays in assembling one of the essential OMPs, namely LptD or BamA. Nevertheless, the fact that BamA and BamD can be expressed separately and recombined into a functional five-protein complex suggests that these proteins do have separate functions that need to be coordinated (63). Since BamC and E stabilize the BamA-D interaction, they may thereby help to tightly couple their functions.

The questions of how the orientation of OMPs in the membrane is determined or of how oligomeric OMPs are assembled have not been addressed. OMPs are asymmetric and contain long hydrophilic loops on the outside of the membrane and short turns on the inside. Their orientation could be determined by the direction in which they are delivered to the Bam complex; the N- and C-termini of OMPs are always found on the inside of the membrane, so if BamA recognizes substrates and initiates folding from the termini, their orientation could be simultaneously established. Alternatively, perhaps the long extracellular loops are specifically directed through the BamA β-barrel in a manner similar to how FhaC secretes its substrate using a flexible loop in its own β-barrel domain (118). This possibility would allow the hydrophilic loops to reach their final destination without needing to pass through the hydrophobic membrane.

It is not clear whether the Bam complex participates in the assembly of folded OMP monomers into their oligomeric forms or whether folded monomers diffuse within the membrane until they encounter and associate with another monomer. The latter possibility does not require that the Bam complex consecutively fold multiple monomers of the same protein, which would require coordination in the periplasm. Furthermore, there is evidence for the existence of folded monomers of proteins that go on to become trimeric in their native states (38). On the other

hand, it appears that no process in the cell is unregulated and more work needs to be done to understand whether the Bam complex coordinates this process or not.

Our understanding of the mechanism of assembly of β-barrel proteins is clearly not at the level of that of α-helical membrane proteins. The biochemical studies described in the next two chapters allow this inherently complex, multi-step process to be studied in isolation using a minimal set of components and allow the roles of those components to be separated. Structural studies of the Sec machine significantly advanced the study of inner membrane protein assembly, and similar structural information about how the Bam proteins interact with each other and with substrates may be required to discern how the Bam complex functions. Chapter 4 describes efforts towards obtaining such information. Identifying specific interactions or steps in the assembly process by these methods could allow us to design ways of disrupting OMP assembly in cells. The dearth of antibiotics available to treat Gram-negative infections and the essentiality and surface location of the Bam complex make it an attractive new target and underscore the necessity of learning its mechanism.

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Chapter 2: Reconstitution of Outer Membrane Protein Assembly from Purified Components

2.1 Why Reconstitute?

Identifying the machinery responsible for assembling β-barrels in bacteria required a combination of bioinformatic, genetic, and biochemical tools. However, once the components of the Bam complex were known, it was not clear how to study their function. Genetic deletions and depletions of any of the Bam components produce pleiotropic phenotypes associated with a defective membrane, including the accumulation of unfolded OMPs and the induction of the σ^E stress response. The individual roles of the Bam proteins in the assembly mechanism cannot be clearly distinguished in these experiments. In an in vitro reconstitution, the Bam proteins can be altered individually without any associated lethality or indirect effects complicating the interpretation of their functions. Moreover, only in an in vitro system can the minimal requirements for β-barrel assembly be identified. The mechanism of β-barrel assembly is believed to be similar in all organisms because the β-barrel assembly machines all contain orthologs of BamA; however, these machines also contain components that are not universally conserved. By understanding the contributions of the different components to the assembly mechanism, the general principles that guide the assembly of all β-barrels can be elucidated.

The design of a reconstitution of β-barrel assembly was guided by earlier reconstitutions of the Sec machine, which translocates proteins across the inner membrane, and of the Lol machine, which transports lipoproteins from the IM to the OM (1-5). These reconstitutions illustrated the importance of identifying a soluble form of the substrate protein and of developing a clear method of monitoring the success and specificity of the process. The identifications of

trigger factor and SecB, chaperones that deliver proteins from the ribosome to the Sec machine, were key advances that dramatically facilitated the translocation of proteins in vitro (6, 7). The ability of LolA, a periplasmic chaperone, to form stable, soluble complexes with OM lipoproteins made it possible to study both the extrusion of OM lipoproteins from the IM and their transfer to the receptor protein, LolB, in the OM (8, 9). The ability to isolate a stable, soluble species makes it possible to monitor steps in the process. In each case, these reconstitutions also established that the activity observed in vitro mimicked the cellular process by demonstrating that it depended on specific components of the Sec and Lol machinery and on the addition of adenosine triphosphate (ATP). This is an essential requirement for any reconstitution; it must recapitulate the cellular process to be a useful analytical tool.

2.2 Over-expression and Purification of the Bam complex

The purification of milligram quantities of homogeneous Bam complex enabled in vitro studies of the complex's activity. Pull-down experiments had demonstrated that three of the lipoproteins, BamC, D, and E, bind only to the fifth POTRA domain of BamA and that BamB dissociates from the complex if any of POTRA domains 2-5 are deleted (10). Dr. Seokhee Kim used this information to develop methods to over-express and purify the five-protein Bam complex and several subcomplexes. Homogeneous five-protein complex was purified by over-expressing BamA and B in one strain and BamC, D, and E in a second strain and then reconstructing the five-protein complex in vitro after lysing the cells and solubilizing their membranes. (See section 2.8.3 for a complete description of the purification methods.) The reconstructed complex was identical to the native complex on a blue native gel (Figure 2.1A). The Bam complex does not dissociate into its components upon blue native gel electrophoresis

and thus runs as a single band at a molecular weight of approximately 230 kilodaltons (kDa). Size exclusion chromatography-light scattering analysis more accurately determined that the over-expressed and reconstructed complex has a molecular weight of 190 kDa, which is too small to accommodate more than one copy of BamA. Quantitative amino acid analysis of the Bam components in the purified complex then indicated that it also contains only one copy of BamB, C, and D, and one or two copies of BamE. Therefore, the stable form of the Bam complex in detergent is monomeric. It is possible that the complex associates to form higher order oligomers in vivo, but the ratio of BamA:B:C:D would still be expected to be 1:1:1:1 (11).

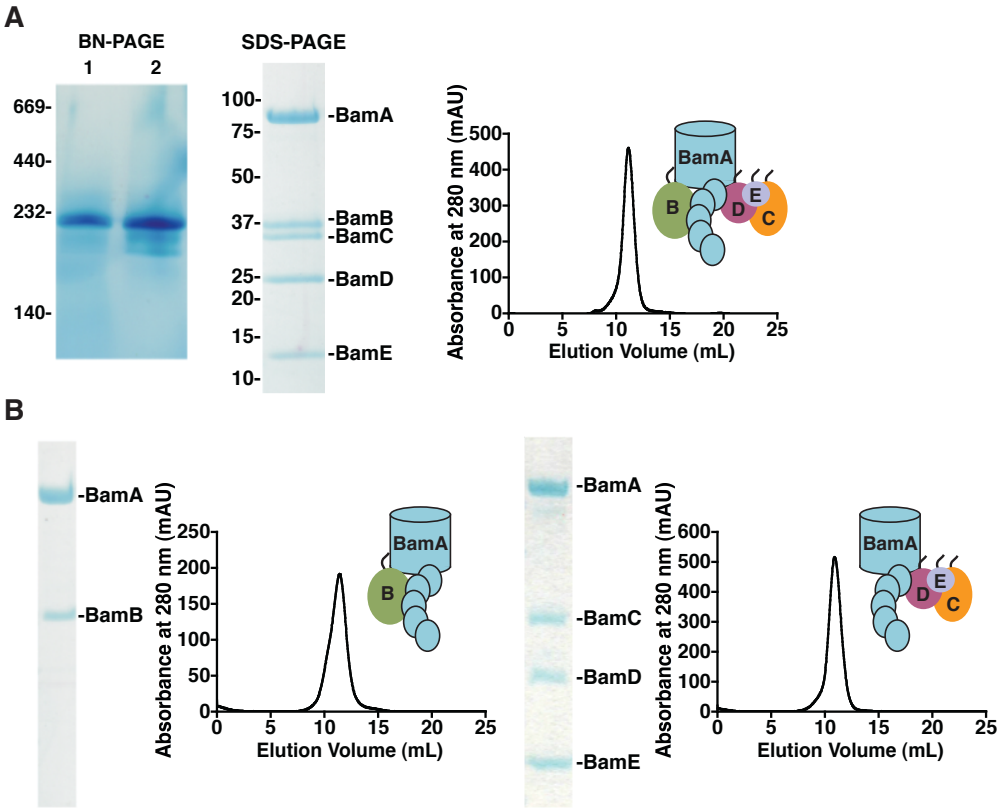

Figure 2.1. The Bam complex and two subcomplexes can be purified. **A.** Blue native gel analysis, sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), and size exclusion chromatogram of the purified five-protein Bam complex. The complex isolated from cells expressing the Bam proteins at basal levels (lane 1) has the same mobility on BN-PAGE as the over-produced and reconstructed complex (lane 2)[1]. **B.** SDS-PAGE and size exclusion chromatograms of the BamAB and BamACDE subcomplexes suggest that they have similar stability to the five-protein complex.

[1] Dr. Seokhee Kim generated the data shown in Figure 2.1A.

Subcomplexes containing two or four proteins, BamAB and BamACDE, could also be purified using similar methods (Figure 2.1B). By comparing the activities of these complexes, it might be possible to determine the roles of the different lipoproteins. The fact that the two essential components of the complex, BamA and BamD, can be over-expressed separately suggests that these proteins may have separate functions. Their essentiality makes it impossible to delete them genetically and determine their individual effects, but an in vitro assay for β-barrel assembly could potentially distinguish their functions.

2.3 Identification of a Folding-Competent Substrate

The activity of the purified Bam complex in assembling OMPs in vitro was initially expected to be very low. It was not known whether removing the Bam complex from its native membrane environment would lower or eliminate its activity by altering its structure, by removing critical, unknown components (e.g. lipids or other proteins), or by decoupling it from earlier steps in the biogenesis pathway. Furthermore, β-barrel membrane proteins are inherently prone to misfolding and aggregation because of their hydrophobicity and ability to form multimers by association of the β-strands of different monomers to form a larger β-sheet. These processes compete with the folding pathway and could make it difficult to observe any activity of the Bam complex. Consequently, an assay that was highly sensitive to the production of a folded β-barrel was needed.

A coupled assay was designed in which an OMP with enzymatic activity was chosen as the substrate. OmpT is a 10-stranded β-barrel that has proteolytic activity when it is folded and associated with lipopolysaccharide (LPS); it cleaves peptides containing consecutive basic residues and its activity can be monitored using a fluorogenic peptide substrate (12). The

fluorogenic substrate is a pentapeptide, *o*-aminobenzoyl-alanine-arginine-arginine-alanine-3-nitrotyrosine-amide, which OmpT cleaves between the two arginine residues. This cleavage separates the aminobenzoyl fluorophore and 3-nitrotyrosine quencher, resulting in increased fluorescence at 430 nm. It was hypothesized that if the purified Bam complex could convert any unfolded OmpT to folded OmpT, it would then become active in the presence of LPS and begin cleaving the fluorogenic peptide to produce a fluorescent signal. The fluorescent signal would thereby report on the activity of the Bam complex indirectly but with high sensitivity such that low levels of folding could be observed. This assay provides a simple method of monitoring the conformational change that the Bam complex induces in its substrates—it couples it to a chemical change (a bond cleavage), which is easily detected.

Two additional issues needed to be addressed: (1) the unfolded OmpT substrate had to be delivered to the Bam complex in a folding competent state; and (2) the background rate of uncatalyzed folding of OmpT had to be minimized. The first issue was addressed by making use of cellular chaperones that are believed to deliver OMP substrates to the Bam complex. SurA affects the transport of the most abundant OMPs and was used to maintain unfolded OmpT in a soluble, folding-competent state in vitro. The second issue reflects the fact that β-barrels are very stable structures and consequently can fold without the aid of the Bam complex into detergent or lipid bilayers (13, 14). This spontaneous process cannot be completely eliminated, but the Bam complex was incorporated into liposomes made of *E. coli* phospholipids to mimic its native membrane environment and to reduce the efficiency of uncatalyzed assembly at least relative to that in a detergent solution. A reconstitution of the machinery that builds the pili structures on the surface of *E. coli* was successfully accomplished in a detergent solution, but the Bam complex did not demonstrate any appreciable activity in detergent (data not shown) (15).

Presumably because the Bam complex catalyzes a folding and membrane insertion process, a membrane is required to observe its activity.

The OmpT substrate was prepared in 8 M urea and then diluted into a solution of SurA. The SurA-OmpT complex was then added to solutions containing the Bam proteoliposomes, the fluorogenic peptide substrate of OmpT, and LPS. The resulting fluorescent signal increased with the concentration of SurA and when the Bam complex was present (Figure 2.2A). These results provided the first evidence that the purified Bam complex affects the assembly of an OMP in vitro.

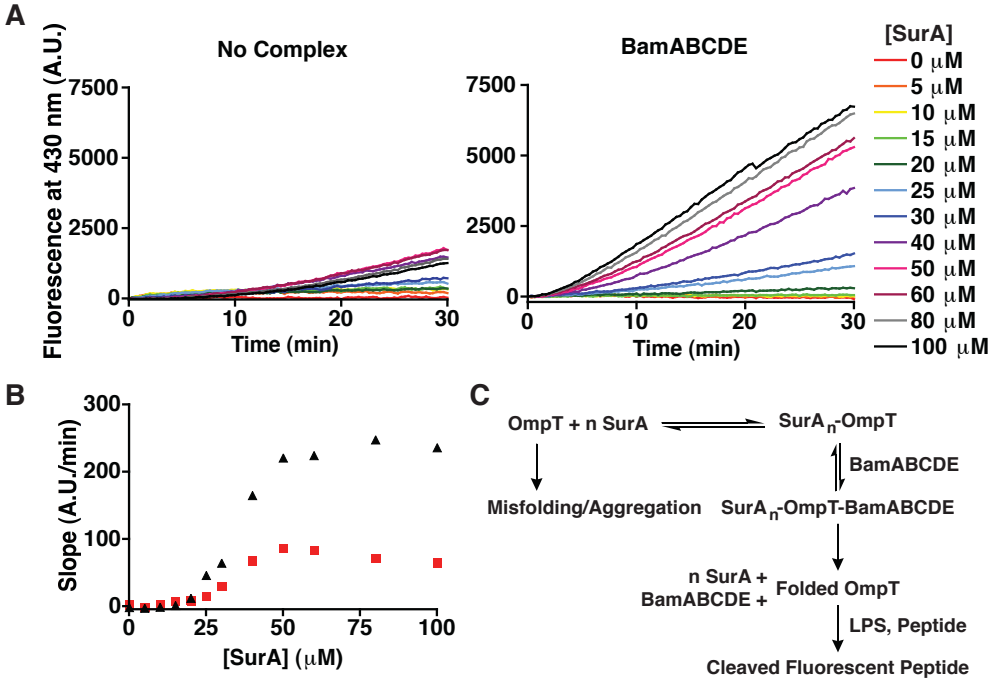

Figure 2.2. SurA and the Bam complex facilitate OmpT assembly in proteoliposomes. **A.** Preincubated solutions of urea-denatured OmpT with SurA were diluted (at time = 0 min) into liposomes (left) or proteliposomes containing the Bam complex (right). The final concentration of OmpT in each reaction was 10 μM, and the final concentration of SurA was varied from 0 to 100 μM. **B.** The amount of active OmpT produced in each reaction in **A** (as determined by the slope between 15 and 30 min) as a function of SurA concentration in the presence (black triangles) or absence (red squares) of the Bam complex. **C.** Schematic of the reconstitution reaction pathway.

Because the assembly process in these experiments is monitored by a coupled assay in which the first step produces the enzyme required for the second step, the first derivative of the fluorescent signal reflects the amount of folded OmpT present in the reaction at any given time.

The slope of the curves in Figure 2.2A becomes constant after approximately 15 minutes, indicating that the concentration of folded OmpT is no longer changing. These slopes (between 15 and 30 minutes) correspond to the total amount of OmpT folded in each reaction, and plotting them against the concentration of SurA demonstrates that there is a sigmoidal relationship between the amount of chaperone and the amount of folded OmpT produced (Figure 2.2B). OmpT activity saturates at approximately the same concentration of SurA regardless of whether the Bam complex is present in the reaction; therefore, the chaperone generally improves the folding competence of the OmpT substrate in a manner that is not dependent on the Bam complex. The concentration at which SurA provided a large increase in OmpT assembly (above 20 μM) is comparable to the binding constants (1 to 14 μM) that have been reported for the interaction of model peptides with SurA, and OmpT contains several putative (Ar-X-Ar) SurA binding sites (16-19). The sigmoidal relationship may indicate that multiple SurA molecules are required to generate a folding-competent $SurA_n$-OmpT complex. The involvement of multiple chaperone molecules might make it possible for an OMP to begin folding on the Bam complex while some chaperone molecules are still associated with its unstructured regions, thereby protecting it from aggregation during the folding process. Nevertheless, the improvement in OmpT activity with SurA concentration indicated that these proteins interact in a functional manner in vitro that mimics their expected behavior in the OMP biogenesis pathway.

2.4 In Vitro Assembly of OmpT Requires the Bam Complex

The final yield of folded OmpT produced in the presence of the Bam complex is only two to three times larger than that produced in its absence (Figure 2.2B). It was therefore necessary to determine whether the Bam proteins were responsible for the observed activity or if there was

some physical property that differed between the empty liposomes and the proteoliposomes. The activities of the Bam subcomplexes were compared to that of the complete, five-protein complex in proteoliposomes prepared by the same methods (Figure 2.3A). The components of these subcomplexes were incorporated into the proteoliposomes in equal proportion to those of the five-protein complex. Cleavage of the fluorogenic peptide was greatly reduced in reactions containing the BamAB and BamACDE subcomplexes compared to those containing the five-protein complex. The four-protein complex lacking BamB consistently demonstrated lower activity than the five-protein complex, and removing the first four POTRA domains of BamA in the BamACDE subcomplex did not dramatically alter its low level of activity (Figures 2.3B and C). Therefore, the folding of OmpT is dependent upon specific components of the Bam complex (*e.g.* BamB), not simply a physical property of the proteoliposomes.

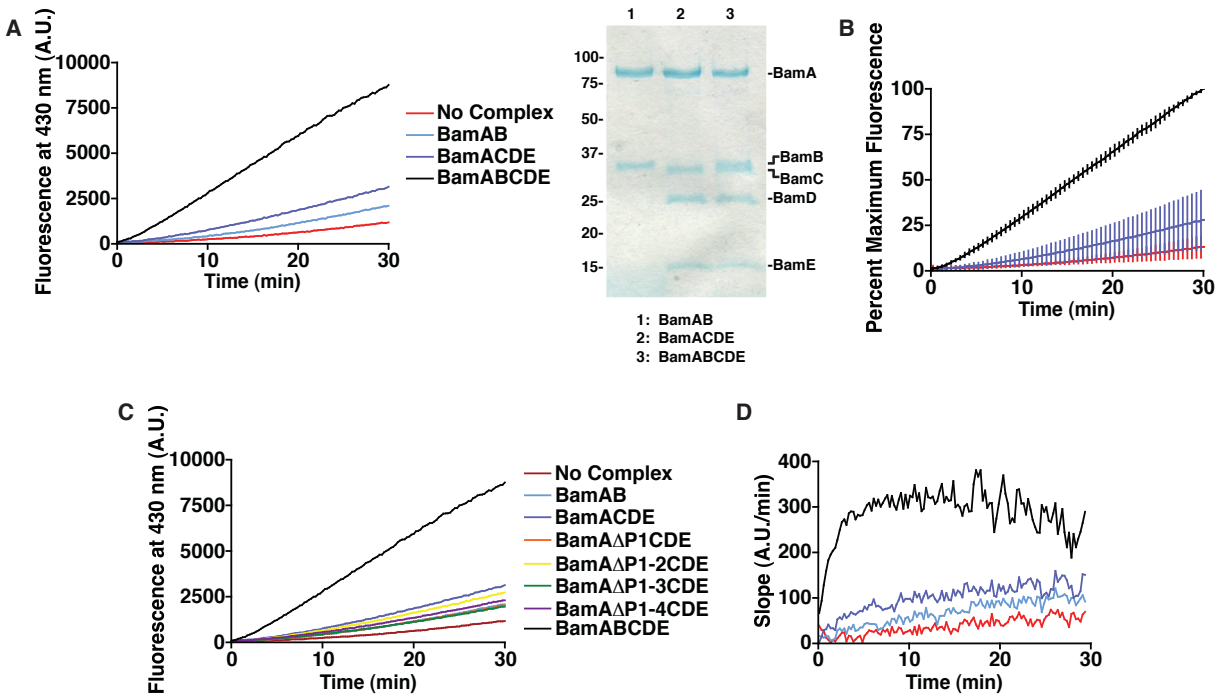

Figure 2.3. OmpT assembly requires specific components of the Bam complex. **A.** Fluorescence produced upon dilution of SurA-OmpT into proteoliposomes containing the Bam complex or subcomplexes (left). OmpT and SurA are diluted to final concentrations of 10 μM and 100 μM, respectively. SDS-PAGE of these proteoliposomes indicates that they contain equal amounts of the appropriate proteins (right). **B.** The BamACDE complex is significantly less active than the BamABCDE complex. Eight different experiments were normalized to their maximum fluorescence values and then averaged. The error bars represent the standard deviation among these experiments. **C.** The first four POTRA domains of BamA do not dramatically affect the activity of the BamACDE subcomplex. **D.** The gradient of the fluorescence data in **A** indicates that the BamABCDE complex rapidly assembles OmpT in the first five minutes of the experiment.

The first derivative of the fluorescence produced over time indicates that, in the first five minutes of the experiment, the rate of assembly of OmpT in the presence of the Bam complex is greater than that in its absence by several orders of magnitude (Figure 2.3D). The BamACDE complex appears to have some activity on this time scale, which BamB dramatically improves. The rates of assembly of a few of the major OMPs have been characterized in vivo in pulse-chase experiments; these studies suggest that OMP assembly occurs on a time scale of 30 seconds to several minutes (20, 21). The fact that the in vitro assembly of OmpT occurs similarly rapidly suggests that the Bam complex can function properly in the proteoliposomes. Moreover, the dependence of this dramatic rate enhancement on the presence of BamB is

consistent with this protein's hypothesized role in vivo. In vivo, SurA and BamB are believed to play related but not redundant roles in OMP biogenesis. Genetic deletions of SurA and BamB affect the kinetics of the same steps in the assembly of LamB, and deleting both proteins produces a synthetic phenotype that is much more severe than that of the single deletions (21-23). Therefore, the importance of BamB in vitro may be related to delivery of OmpT to the Bam complex with SurA. These proteins may function in the same pathway to improve the efficiency of assembly, and the highly simplified, reconstituted system recapitulates their cellular roles.

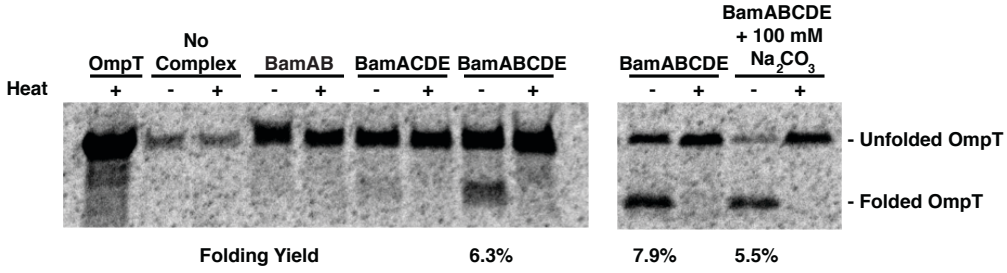

Figure 2.4. OmpT assembled by the Bam complex is folded on SDS-PAGE and resistant to membrane extraction. The pellets of reactions of SurA and ^{35}S-labeled OmpT with the Bam proteoliposomes were run on SDS-PAGE with and without prior boiling and then visualized by autoradiography. OmpT and SurA were diluted to final concentrations of 0.4 μM and 100 μM, respectively. Washing the BamABCDE reaction pellet with 100 mM sodium carbonate results in a three-fold enrichment of the folded material relative to the unfolded material.

To verify that fluorescence reported accurately on the assembly of OmpT, a more direct assay was developed. ^{35}S-labeled OmpT was purified, incubated with SurA, and diluted into the Bam proteoliposomes as in the fluorescence experiments. After 30 minutes, the proteoliposomes were centrifuged, and the unfolded and folded forms of OmpT in the pellet were separated by SDS-PAGE. The stability of β-barrels prevents them from unfolding in SDS unless they are boiled; consequently, the folded forms of these proteins migrate faster on SDS-PAGE than their unfolded forms. This type of analysis has been termed semi-native SDS-PAGE (24). As expected, folded OmpT was observed on the gel following incubation with the BamABCDE complex, and a small amount was produced by the BamACDE subcomplex (Figure 2.4). The

material in the faster migrating band was denatured by boiling and resistant to extraction from the proteoliposome pellet with sodium carbonate, which suggests that it is both folded and inserted into the membrane. (Sodium carbonate has been shown to remove proteins that are peripherally associated with the surfaces of membranes but not those that are integrated into the membrane (25, 26).) Although the amount of OmpT folded by the complete complex is low (approximately seven percent of the substrate as determined by five separate experiments), this material bears the important properties of properly assembled OMPs. Therefore, the activity of the Bam complex has been successfully reconstituted in vitro.

2.5 Optimization of OmpT Assembly by the Bam Complex

The low level of activity of the Bam complex described in the previous section was sufficient to demonstrate that it can assemble an OMP in vitro, but more detailed mechanistic studies require higher activity in order to observe the effects of small changes in components of the complex. Lipopolysaccharide is required for OmpT protease activity, but it is not required for OmpT assembly. When LPS was omitted from the assembly reactions, the folding yield increased ten-fold (27). With a yield of approximately 70 percent, it became possible to monitor the assembly of OmpT over time directly on semi-native SDS-PAGE. Folded OmpT accumulates on the same time scale as that observed in the fluorescent assay; in the presence of the five-protein complex, folding occurs rapidly in the first two minutes of the experiment and is virtually complete after ten minutes (Figure 2.5).

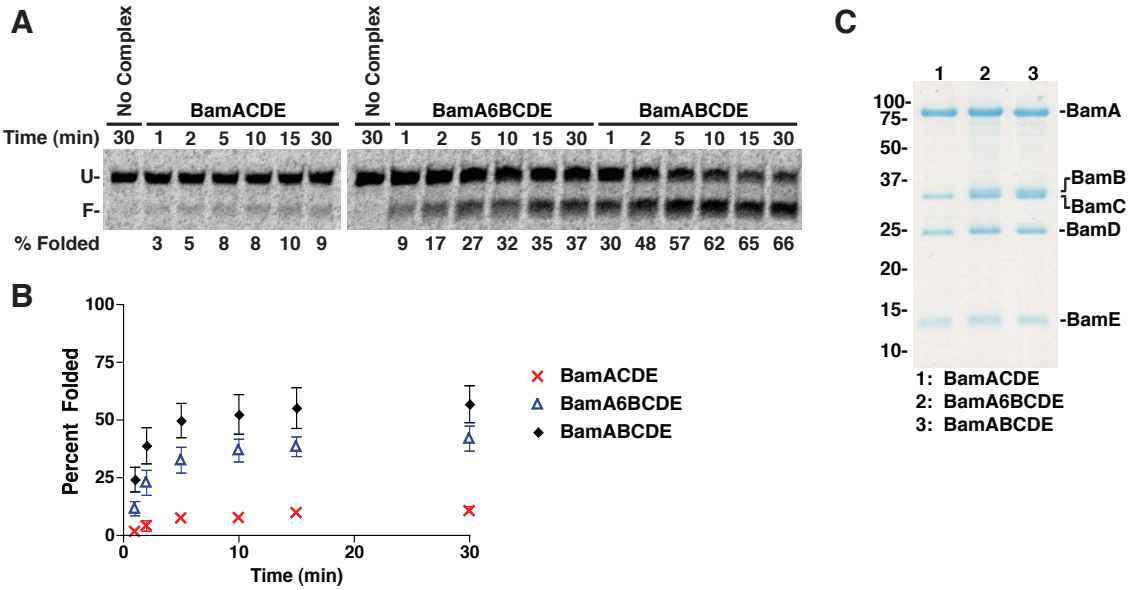

Figure 2.5. Folding of ^{35}S-labeled OmpT by wild-type and deficient Bam complexes can be monitored directly. **A.** Autoradiogram of the time course of OmpT folding in proteoliposomes containing BamACDE, BamA6BCDE, or BamABCDE. Folding reactions were stopped at the indicated time points and run on SDS-PAGE without prior boiling. (U: unfolded OmpT, F: folded OmpT) **B.** Plot of the average yield of OmpT produced over time in three separate experiments like that in **A**. The error bars indicate the standard deviation among these experiments. **C.** SDS-PAGE of the proteoliposomes used in these experiments indicates that they contain equal amounts of the relevant Bam components.

Using these improved assay conditions, it was possible to observe the effect of a two amino acid insertion in BamA on the assembly of OmpT. This mutation (denoted *bamA6*) was isolated as a suppressor of the sensitivity of an *lptD4213* strain to bile salts and is a duplication of glutamine 217 and lysine 218 in a long loop in the third POTRA domain of BamA (28). This loop is longer than the corresponding loops in any of the other POTRA domains, and crystal structures of the POTRA domains suggest that it may be conformationally flexible (10, 29). This region of the protein may be mechanistically important, as the *bamA6* mutation decreases the levels of assembled OmpA and LamB in the OM by approximately ten percent (28). However, the *bamA6* mutation induces the σ^E stress response, which decreases the synthesis of OMPs; therefore, the direct and indirect effects of the mutation cannot be separated in vivo. In vitro, a five-protein complex containing this mutation (BamA6BCDE) assembles less OmpT than the

wild-type complex but more than the BamACDE complex—in accord with the more severe decrease in OMP levels associated with a *bamB* null mutation. The reconstituted system thus makes it clear that these mutations directly affect the activity of the Bam complex. Because the Bam proteoliposomes are identical in their preparation and composition and the substrate is delivered to them in an identical manner, the effect of the two amino acid insertion on the mechanism of the Bam proteins is unambiguous.

The concentrations of ^{35}S-labeled OmpT used in the experiments described thus far have been sub-saturating—that is, the concentration of Bam complex exceeds that of the substrate. In order to assess the catalytic efficiency of the Bam complex, larger quantities of substrate must be used. However, the main problem in attaining higher yields of folded protein in the reconstituted system is maintaining the folding competence of the substrate. Although folding stops approximately ten minutes into the reaction, the Bam complex remains active (Figure 2.6A). The remainder of the OmpT substrate, therefore, must not be foldable. The effects of SurA and urea on the reaction were examined in greater detail in order to achieve higher levels of conversion of unfolded OmpT to folded OmpT.

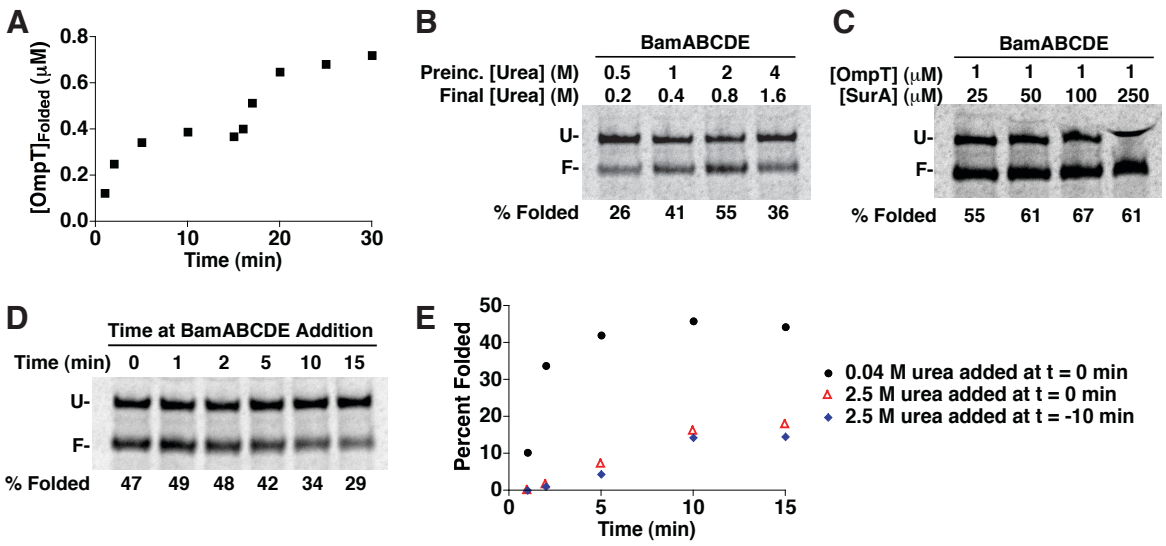

Figure 2.6. SurA and urea each contribute to the folding competence of the OmpT substrate. Urea-denatured OmpT was preincubated with a 25-fold excess of SurA (except as noted in panel **C**) and then diluted into solutions containing BamABCDE proteoliposomes. Reactions were stopped after 30 minutes unless indicated otherwise. **A.** Folding stops after approximately 10 minutes, but the Bam complex is still active. An aliquot of SurA-OmpT added 15 minutes into the reaction can be folded after folding of the first aliquot (added at time = 0 min) has stopped. The folding yields were determined by autoradiography. **B.** The yield of folded OmpT increases with the concentration of urea used during the preincubation until the final concentration in the reaction exceeds approximately 1 M. **C.** Additional SurA does not significantly improve the folding yield. **D.** The SurA-OmpT substrate loses folding competence over time following dilution. Preincubated SurA-OmpT substrate was diluted into urea-free buffer prior to the addition of the BamABCDE proteoliposomes at the indicated time points. The folding yield decreases with the length of time the substrate was allowed to incubate under dilute conditions. **E.** High concentrations of urea inhibit the activity of the Bam complex. SurA-OmpT was diluted into BamABCDE proteoliposomes without any additional urea, simultaneously with 2.5 M urea, or ten minutes after the addition of 2.5 M urea. The folding yield at the indicated time points was determined by autoradiography. The effect of urea on the Bam complex is rapid; the activity of the complex after ten minutes of incubation in 2.5 M urea is similar to that observed immediately after the urea addition.

When denatured OmpT was incubated with an excess of SurA and increasing concentrations of urea, the folding yield upon dilution into Bam proteoliposomes increased with the urea concentration until it exceeded a final concentration of approximately 1 M (Figure 2.6B). Additional SurA does not significantly improve the folding efficiency, suggesting that urea improves the folding competence of OmpT in a slightly different manner (Figure 2.6C). The interaction between unfolded OmpT and SurA may be dynamic; higher concentrations of urea may help keep the substrate in a foldable state while SurA dissociates and reassociates. Accordingly, when the SurA-OmpT substrate is diluted into urea-free buffer, it starts to become

less foldable (Figure 2.6D). However, urea has deleterious effects on the assembly reaction at high concentrations; above 1 M, urea decreases the rate of OmpT folding and the final yield of folded product (Figure 2.6E). Exposing the Bam complex to these concentrations of urea may cause some unfolding or dissociation of the Bam proteins. Alternatively, if the substrate binds to the Bam complex through hydrogen bonding interactions (perhaps, as discussed in Chapter 1, by augmenting the β-strands of the POTRA domains), urea could alter the affinity of those interactions and thereby interfere with the folding process.

2.6 The Bam Complex Functions Catalytically In Vitro

Given these constraints, the ability of the Bam complex to perform multiple turnovers was determined by adding higher concentrations of SurA-OmpT while keeping the final concentration of urea under 1 M. The folding yield increases linearly with the substrate concentration under these conditions, and at the highest concentrations, the amount of folded OmpT exceeds the amount of Bam complex present in the reactions (Figure 2.7). Therefore, an individual Bam complex is capable of folding multiple OmpT molecules in vitro. If all of the Bam complexes in the proteoliposomes are active, they perform approximately 1.6 turnovers in this experiment. However, it is possible, or even likely, that not all of the complexes are active and a smaller percentage of them are performing a larger number of turnovers. In initial reconstitutions of the Sec machine, approximately 15% of the reconstituted complexes were active, but they were capable of performing approximately 22 rounds of protein translocation (30). The number of active Sec machines was determined by using a substrate protein that could not be completely translocated and then quantifying the number of these proteins trapped in Sec complexes. A method of generating a stable interaction between a substrate and the Bam

complex has not yet been developed, so it was not possible to determine the percentage active Bam complexes in an analogous manner. However, it is clear that the reconstituted complex can complete the assembly of a β-barrel without any additional cellular components.

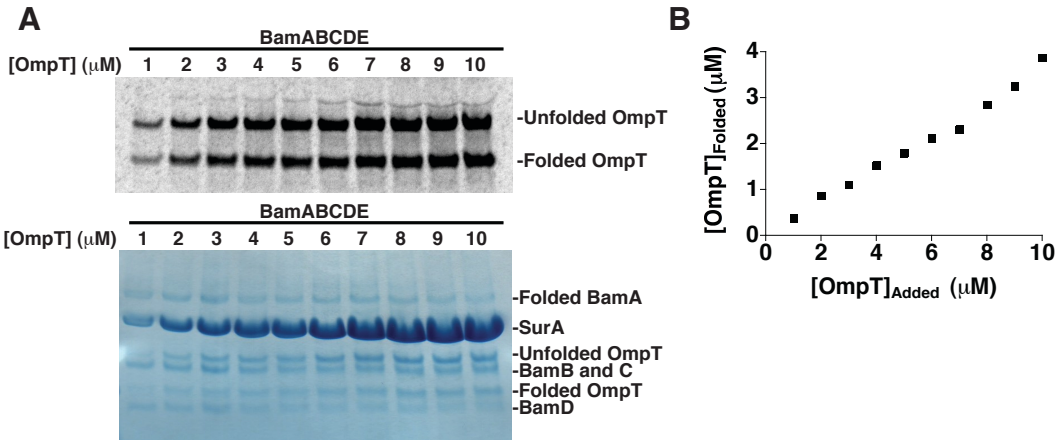

Figure 2.7. The reconstituted Bam complex performs multiple rounds of β-barrel assembly. **A.** Autoradiogram and coomassie blue stained gel of folding reactions containing increasing amounts of SurA-OmpT substrate. OmpT was preincubated with a ten-fold excess of SurA and then diluted into solutions containing approximately 2.5 μM BamABCDE in proteoliposomes. Reactions were stopped after 30 minutes. At the highest substrate concentrations, the amount of folded OmpT produced exceeds the amount of BamABCDE in the reaction mixture. (Compare the levels of folded OmpT and BamD in the stained gel.) **B.** The yield of folded OmpT produced in the reactions in **A** increases linearly with substrate concentration. The yield at the highest tested substrate concentration corresponds to approximately 1.6 turnovers of the Bam complex.

2.7 Conclusion

The reconstitution of a cellular process provides a direct demonstration of the function of the component proteins. The results presented in this chapter demonstrate that the Bam complex folds and inserts a β-barrel without any external energy input. It dramatically increases the rate of OmpT assembly in vitro compared to the uncatalyzed process; therefore, the Bam complex changes the folding and insertion pathway of OMPs. The energy independence of this process was not unexpected—the periplasm and OM do not contain any obvious energy sources (e.g. ATP, membrane potential)—but it is surprising. The only other cellular complex that assembles integral membrane proteins, the Sec machine, uses the hydrolysis of ATP to drive protein

translocation and membrane insertion. The barriers to OMP assembly may include organizing the β-strands of a substrate into a barrel such that the N- and C-termini are adjacent, moving the hydrophilic extracellular loops of the barrel across the hydrophobic membrane, and reorganizing the lipids in the membrane to allow the protein to insert. It is not obvious how the Bam proteins could facilitate these processes, but their structures must provide a scaffold that inherently does so. The crystal structures of the POTRA domains of BamA suggest that P3 may initiate β-barrel assembly by interacting with substrates by β-strand augmentation. The reconstitution has validated the importance of this domain (although not specifically the β-strand augmentation mechanism) by demonstrating that a two amino acid insertion in this domain decreases the efficiency of OMP assembly. By binding substrates and acting as a template for β-strand formation, the POTRA domains could offset some of the entropic cost of folding the substrate and thereby provide a different pathway for β-barrel assembly.

The SDS-PAGE assay described in this chapter can, in principle, be adapted to study the assembly of any OMP. The conditions of the assay no longer require the high sensitivity that the enzymatic activity of OmpT provided. Chapter 3 describes the use of this more general assay to study the assembly of the central component of the Bam complex, BamA.

2.8 Materials and Methods

2.8.1 Materials

All vectors used for plasmid construction were obtained from Novagen, and restriction enzymes were purchased from New England BioLabs. Unless noted otherwise, all cultures were grown in LB media from Difco with the appropriate antibiotics added at concentrations of 50 μg/mL. The n-dodecyl-β-D-maltopyranoside (DDM) and lauryldimethylamine-N-oxide (LDAO) used in the Bam complex purifications were purchased from Anatrace, and the tris(2-carboxyethyl)phosphine (TCEP) was purchased from Hampton Research. The Ni-NTA and TALON resins were purchased from Qiagen and Clontech, respectively. All size exclusion chromatography steps were performed using a Superdex 200 column from GE Healthcare. The *E. coli* phospholipids used in preparing the proteoliposomes were obtained from Avanti Polar Lipids as a dried polar lipid extract, and the lipopolysaccharides used in the folding assays were purchased from Sigma as an ion-exchange purified extract from *E. coli* 0111:B4. The fluorogenic peptide, Abz-Ala-Arg-Arg-Ala-Tyr(NO_2)-NH_2, was obtained from New England Peptide. The [^{35}S]-methionine (>37 TBq/mmol) used to label the His_6-OmpT was purchased from American Radiolabeled Chemicals, and the EZ Rich media lacking methionine was purchased from Teknova. All protein concentrations were determined using the Bio-Rad DC Protein Assay unless noted otherwise.

2.8.2 Plasmid Construction

The plasmids used for over-expression of the proteins in this study are described in Table 2.1. All genes were amplified from MC4100 chromosomal DNA using the primers listed in Table 2.2. The restriction sites highlighted in bold were used to construct the indicated plasmids.

The *bamA6* mutation was introduced into pSK38 by site directed mutagenesis; the inserted nucleotides are highlighted in bold in the primers used in this procedure.

2.8.3 Expression, In Vitro Reconstruction, and Purification of the Bam complex

BamA and BamB were over-produced in BL21(DE3) cells by growing the strain carrying pSK38 to OD_{600} = 0.2-0.3 at 37 °C. The growth temperature was shifted to 25 °C over 30 minutes, and the over-expression was then induced by addition of IPTG (0.1 mM) when the OD_{600} = 0.5-0.6. The cultures were grown at this temperature for an additional 3-4 hours. BamC, D, and E were co-expressed by transforming BL21(DE3) with both pSK46 and pBamE-His. (The BamE-His plasmid was previously described by Sklar *et al*. as pSmpA-His (31).) Cultures of this strain were grown to OD = 0.5-0.6 at 37 °C and then induced with 0.1 mM IPTG. The incubation continued for 3-4 hours. Cells from the two strains were harvested and resuspended in 20 mM Tris-HCl (pH 8) buffer. They were lysed by French press and centrifuged at 5,000 x g for 10 min at 4 °C to remove unbroken cells and debris. The supernatants were ultracentrifuged at 100,000 x g for 30 min to pellet the membranes, which were then solubilized in TBS (pH 8) (*i.e.* 20 mM Tris (pH 8), 150 mM NaCl), 2% Triton X-100, 10 mM EDTA, and 0.1 mg/ml lysozyme for 30 minutes at room temperature. The solutions were then ultracentrifuged again, and the supernatants were dialyzed overnight against TBS (pH 8), 0.5% Triton X-100 to remove the EDTA. Small amounts of the dialyzed BamAB and BamCDE solutions were mixed in increasing AB:CDE ratios. These were then purified by Ni-NTA affinity chromatography. The eluted proteins were run on SDS-PAGE, and the ratio of BamAB to BamCDE that produced equal amounts of BamB and BamC in the eluate was selected for the reconstruction of the complex. The large scale reconstruction was carried out

and the eluate of the Ni-affinity purification was concentrated and subjected to size exclusion chromatography in TBS (pH 8), 0.03% DDM, and 1 mM TCEP. The Bam complex containing the *bamA6* mutation was purified by the same methods using a BL21(DE3) strain carrying pch114 instead of pch38.

2.8.4 Characterization of the Purified Bam Complex by Blue Native Electrophoresis

Seokhee Kim compared the mobility on a blue native gel of the over-expressed and reconstructed complex to that of the complex isolated from cells expressing the Bam proteins at basal levels (Figure 2.1A). The electrophoresis was carried out on a 4-20% gradient gel as previously described (10, 32). The native complex was isolated by a procedure described by Malinverni *et al* (33). Briefly, the strain NR721 (MC4100 *yfgL::kan*) was transformed with a plasmid (pTW006) encoding YfgL-His_6. Cultures of this strain were grown overnight at 37 °C and then pelleted at 5,000 x g for 10 min at 4 °C. The cells were lysed by French press and the membranes were collected and solubilized as described for the over-expressed complex. The resulting solution was then subjected to Ni-NTA affinity chromatography, and the detergent was exchanged to 0.05% DDM in this step. The eluted complex was run on BN-PAGE.

2.8.5 Expression and Purification of the Bam Subcomplexes

2.8.5.1 BamAB

Cultures of BL21(DE3) cells carrying pSK86, which encodes *bamB* and *bamA* with an N-terminal His-tag on a pETDuet vector, were grown and induced in the same manner as the strain carrying pSK38 described in the purification of five-protein complex. The cells were harvested and lysed by French press, and the membranes were collected and solubilized in Triton X-100

according to the same methods. After overnight dialysis of the membrane solutions, the two-protein complex was purified by Ni-NTA affinity chromatography in TBS (pH 8), 0.05% DDM. The His-tag on BamA was then removed by digestion with thrombin for ~24 hours. Residual His-tagged protein was removed by Ni-NTA affinity purification, and the digested material was further purified by size exclusion chromatography in TBS (pH 8), 0.03% DDM, 1 mM TCEP.

2.8.5.2 BamACDE and Truncated BamACDE

N-terminally His-tagged BamA and BamA variants containing serial POTRA deletions, BamAΔP1 - BamAΔP1-4, were expressed as inclusion bodies in BL21(DE3) transformed with pSK52 and pSK136 - pSK139, respectively. Cultures of these strains were grown at 37 ^{o}C to OD_{600} = 0.4. Expression of the BamA proteins was then induced by addition of 0.1 mM IPTG, and the cultures were incubated for another 3-4 hours. The cells were harvested, resuspended in 20 mM Tris-HCl (pH 8), and lysed by French press. They were centrifuged at 5,000 x g for 10 min, and the pellets were resuspended in 8 M urea. After rocking at room temperature for about an hour, the solutions were centrifuged at 5,000 x g for 10 min. The supernatants contained the denatured His-tagged BamA variants. These urea solutions were diluted ten-fold into TBS (pH 8), 0.5% LDAO and incubated on a rocker at 4 °C overnight to allow the β-barrels of these BamA variants to fold. The solutions were then centrifuged at 5,000 x g for 10 minutes to remove aggregated material. The His-tags on these refolded proteins were removed by thrombin digestion for ~24 hours and the remaining undigested material was removed by Ni-NTA affinity chromatography. The three lipoproteins, BamCDE, were expressed as described in the reconstruction of the five-protein complex, and the solubilized membranes were mixed with the refolded, digested BamA variants. The four-protein complexes were then purified by Ni-NTA

affinity chromatography (using the His-tag on BamE) in TBS (pH 8), 0.05% DDM. Finally, the eluates were concentrated and subjected to size exclusion chromatography in TBS (pH 8), 0.03% DDM, 1 mM TCEP.

2.8.6 Expression and Purification of His_6-SurA

His_6-SurA was expressed in BL21(DE3) carrying pSK257, which encodes the protein without its signal sequence such that it is expressed in the cytoplasm. Cultures were grown at 37 °C to OD_{600} ~ 1, shifted to 16 °C, and then induced with 0.1 mM IPTG and incubated overnight. After harvesting the cells, they were resuspended in 20 mM Tris-HCl (pH 8) and lysed by French press. The lysed cells were centrifuged at 5,000 x g for 10 minutes at 4 ^{o}C, and the His-tagged protein was purified from the supernatant by TALON and subsequent Ni-NTA metal affinity chromatography. The final eluate was concentrated and diluted three times with TBS (pH 8) to remove residual imidazole.

2.8.7 Expression and Purification of OmpT

The purification of an OmpT variant carrying the mutation G236K/K237G, which eliminates autoproteolytic degradation of the protein, has been described previously (12). This variant was expressed without its signal sequence as inclusion bodies in BL21(DE3) carrying pCH18. Cultures were grown to OD_{600} = 0.4 at 37 °C, induced with 0.1 mM IPTG, and incubated for another four hours. The cells were then harvested, lysed by French press, and centrifuged at 5,000 x g for 10 min at 4 ^{o}C. The pellets contained the inclusion bodies; these were washed by resuspension in TBS (pH 8) and pelleted again. The pellets were then resuspended in 8 M urea and incubated at room temperature for approximately one hour. Any

material that did not dissolve was pelleted by centrifugation at 20,000 x g for 20 minutes at 20 °C. The urea solutions contained only minor amounts of other contaminating proteins as judged by SDS-PAGE, and the denatured OmpT was used without further purification.

2.8.8 Expression and Purification of Radiolabeled His_6-OmpT

His_6-OmpT G236K/K237G was expressed as inclusion bodies in BL21(DE3) from pCH28, which encodes this OmpT variant without its signal sequence. Cultures (50 mL) were grown in methionine-free EZ Rich media to OD_{600} = 0.4 at 37 °C at which point the expression of His_6-OmpT was induced by addition of 1 mM IPTG. After 15 minutes of incubation at 37 °C, 2 mCi of [^{35}S]-methionine were added, and the cultures were incubated for an additional three hours. The cells were harvested and resuspended in 8 M urea. After 20 minutes of incubation at room temperature, the cellular debris was pelleted by centrifugation at 16,000 x g, 10 min, 4 °C, and His_6-OmpT was purified from the supernatant by Ni-NTA chromatography under denaturing conditions. After loading the supernatant, the Ni-NTA resin was washed with 8 M urea at pH 6.3 and then at pH 5.9. The His-tagged protein was eluted in 8 M urea, pH 4.5. The protein concentration in the eluate was determined by absorbance at 280 nm using an extinction coefficient of 78270 M^{-1} cm^{-1}.

2.8.9 Proteoliposome Preparation

Proteoliposomes containing the Bam complexes were prepared by detergent dilution methods adapted from procedures described by van der Does *et al*. (34). *E. coli* phospholipids (40 μL of a 20 mg/mL sonicated aqueous suspension) were added to the purified Bam complexes (200 μL of 10 μM solutions) in TBS (pH 8), 0.03% DDM, 1 mM TCEP and incubated on ice for

five minutes. At this point, two slightly different procedures were followed. For the experiments in Figures 2.2-2.4, the phospholipid, detergent, protein complex mixtures were diluted with 4 mL of 10 mM Tris-HCl (pH 6.5) and incubated on ice for 30 minutes. These proteoliposomes were then pelleted by ultracentrifugation at 125,000 x g for 30 minutes at 4 °C, washed by resuspension in 1 mL of 10 mM Tris-HCl (pH 6.5), and ultracentrifuged again at 125,000 x g for 30 min at 4 °C. They were finally resuspended in 200 μL of 10 mM Tris-HCl (pH 6.5). For the experiments in Figures 2.5-2.7, the phospholipid, detergent, protein complex mixtures were diluted with 8 mL of TBS (pH 8), incubated on ice for 30 minutes, and then ultracentrifuged at 300,000 x g for 2 hours at 4 ^{o}C. These proteoliposomes were not washed and were directly resuspended in 200 μL of TBS (pH 8). This latter procedure results in higher levels of incorporation of the Bam complex into the lipid vesicles. Any proteoliposomes that were not used immediately were flash frozen in liquid nitrogen and stored at -80 °C.

2.8.10 OmpT Folding Fluorescent Assay

2.8.10.1 SurA Dependence Assays

The folding reactions were carried out in 100 μL solutions prepared as follows: 25 μL of the proteoliposomes were diluted in 50 μL of 10 mM Tris-HCl (pH 6.5), and the LPS (5 μL of a 5 mg/mL solution) and fluorogenic peptide (10 μL of a 10 mM solution) were then sequentially added. Purified, urea-denatured OmpT was diluted 5-10 fold into solutions of His_6-SurA such that the final concentration of OmpT is 100 μM. These SurA-OmpT solutions were incubated at room temperature for 10 minutes and then diluted 10-fold into the proteoliposome solutions. The fluorescence produced by the reactions was then monitored on a Spectramax Gemini XS plate reader for 30 minutes with readings every 20 seconds. Fluorescence emission was

monitored at 430 nm following excitation at 325 nm, and the background signal produced at the zero time point was subtracted. The final concentrations of the reaction components were 10 μM OmpT, 0-100 μM SurA, ~0.2 μM Bam complex, 0.25 mg/mL LPS, and 1 mM fluorogenic peptide. The slope of the fluorescence curves between 15 and 30 minutes were determined by linear regression and plotted against the concentration of SurA.

2.8.10.2 Bam Complex and Subcomplex Comparative Activity Assays

The folding reactions were prepared as in the previous section with the following exceptions. SurA was used at a final concentration of 100 μM in all of the reactions. Fluorescence was monitored following dilution of the preincubated SurA-OmpT into the proteoliposomes every 15 seconds for 30 minutes. Reactions without OmpT were run in parallel and used to subtract the background signal produced by the liposomes, SurA, LPS, and the fluorogenic peptide. The first derivatives (or gradients) of the fluorescence plots were determined in Prism (GraphPad Software) using a smoothing method that calculates a weighted average of the 13 nearest neighboring points.

2.8.11 [^{35}S]-His-OmpT Folding SDS-PAGE Assay

[^{35}S]-His-OmpT was preincubated with SurA by diluting the purified, urea-denatured OmpT stock into a solution of SurA in TBS (pH 8) and then incubating at room temperature for 10 minutes. In the experiment in Figure 2.4, the concentrations of SurA and OmpT in the preincubation solutions were 100 μM and 0.4 μM, respectively; in Figures 2.5 and 2.6, they were 250 μM and 10 μM, respectively; and in Figure 2.7, they were 320 μM and 32 μM, respectively. These preincubated solutions were then diluted into suspensions of the proteoliposomes (as in

the fluorescence experiments) and incubated at room temperature for the time periods indicated in the figures. In the experiment in Figure 2.4, the reactions were chilled on ice after 30 minutes and then centrifuged at 16,000 x g for 10 min at 4 °C. The pelleted material was resuspended in cold 10 mM Tris-HCl (pH 6.5) and 2% SDS sample loading buffer. The pellet of a separate reaction with the complete Bam complex was resuspended in ice cold 100 mM sodium carbonate (pH 11) (as described by Molloy (26) for isolation of bacterial membrane proteins) and incubated on ice for 30 minutes before being centrifuged again. The pellet after this wash was then also resuspended in 10 mM Tris-HCl (pH 6.5) and SDS sample buffer. Half of each sample of pelleted material was boiled for 10 minutes, and both the boiled and non-boiled samples were run on SDS-PAGE (4-20% gradient gel) at 150 V for 90 minutes at 4 °C. For the experiments in Figures 2.5-2.7, the reactions were stopped by directly adding cold 6x SDS sample buffer (375 mM Tris, pH 6.8, 9% SDS, 60% glycerol, 0.015% bromophenol blue, 12% β-mercaptoethanol); the samples were then applied to SDS-PAGE (4-20% gradient gel), and run at 150 V for 110 minutes at 4 ^{o}C.

The SDS-PAGE gels were dried and visualized by storage phosphor autoradiography on a GE Typhoon Imager. ImageQuant TL was used to calculate the densities of the observed bands. The yields of folded protein in Figure 2.4 were determined by comparison to a standard curve of known amounts of radiolabeled OmpT, and the percent yields in Figure 2.5-2.7 were determined by comparing the densities of the folded and unfolded bands in each lane. Absolute yields were calculated from the percent yields and the known concentrations of OmpT that were added to each reaction.

Table 2.1 Plasmids used in this study

Name	Description	Construction
pSK38	pETDuet-bamB-bamA	PCR with primers: bamB-N, bamB-C and bamA-N, bamA-C
pSK46	pCDFDuet-bamC-bamD	PCR with primers: bamC-N, bamC-C and bamD-N3, bamD-C3
pBamE-His	pET22-42-bamE-His_8	As described by Sklar *et al.* (31)
pTW006	pET23a-yfgL-His_6	As described by Wu *et al.* (35)
pSK86	pETDuet-bamB-His_6-bamA	pSK38 with inserted His-tag and thrombin site after bamA signal sequence
pSK52	pET28b-His_6-bamA (A21-W810)	PCR with primers: bamA-Nns and bamA-Ce
pSK136	pET28b-His_6-bamAΔP1 (E90-W810)	PCR with primers: p2N2 and bamA-Ce
pSK137	pET28b-His_6-bamAΔP1-2 (V173-W810)	PCR with primers: p3N2 and bamA-Ce
pSK138	pET28b-His_6-bamAΔP1-3 (D264-W810)	PCR with primers: p4N2 and bamA-Ce
pSK139	pET28b-His_6-bamAΔP1-4 (G344-W810)	PCR with primers: p5N2 and bamA-Ce
pSK257	pET28b-His_6-surA (A21-N428)	PCR with primers: SurA-Nns and SurA-Ce
pCH18	pET22b-ompT G236K/K237G (S21-F317)	PCR with primers: OmpT-Nns and OmpT-C and site directed mutagenesis with primers described by Kramer *et al*. (12)
pCH28	pET28b-His_6-ompT G236K/K237G (S21-F317)	PCR with primers: OmpT-Nns and OmpT-C and site directed mutagenesis with primers described by Kramer *et al*. (12)
pCH114	pETDuet-bamB-bamA6	Site directed mutagenesis with primers: bamA6-for and bamA6-rev

Table 2.2 Primers used in plasmid construction

Name	Sequence
bamB-N	ACAC**CCATGG**GACAATTGCGTAAATTACTGCTGC
bamB-C	ACAC**GCGGCCGC**TTAACGTGTAATAGAGTACACGGTTC
bamA-N	GTCCTAGAG**CATATG**GCGATGAAAAAGTTGC
bamA-C	ACAC**GACGTC**TTACCAGGTTTTACCGATGTTAAAC
bamC-N	ACAC**CCATGG**GAGCTTACTCTGTTCAAAAGTCG
bamC-C	ACAC**GCGGCCGC**TTACTTGCTAAACGCAGC
bamD-N3	ACAC**CATATG**ACGCGCATGAAATATCTG
bamD-C3	ACAC**GACGTC**TTATGTATTGCTGCTGTTTGC
bamA-Nns	ACAC**CATATG**GCTGAAGGGTTCGTAGTGAA
bamA-Ce	ACAC**GCGGCCGC**TTACCAGGTTTTACCGATGTTAAACTG
p2N2	AGAG**CATATG**GAACGTCCGACCATTGCCAGC
p3N2	AGAG**CATATG**GTGTCAGCTGAAATCCAGCAAATTAAC
p4N2	AGAG**CATATG**GATCAGTACAAGCTTTCTGGCGTTG
p5N2	AGAG**CATATG**GGTAACCGTTTCTACGTGCGTAAG
SurA-Nns	ACAC**CATATG**GCCCCCCAGGTAGTCGATAAAG
SurA-Ce	ACAC**GCGGCCGC**TTAGTTGCTCAGGATTTTAACGTAGG
OmpT-Nns	ATGA**CATATG**TCTACCGAGACTTTATCGTTTACTCCTGACAACATAAATGC
OmpT-C	ATTA**GCGGCCGC**TTAAAATGTGTACTTAAGACCAGCAGTAGTGATGAA
bamA6-for	CAGAAA**CAGAAA**CTGGCGGGCGACCTTG
bamA6-rev	CCCGCCAG**TTTCTG**TTTCTGTTTCTGGTATT

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Chapter 3: In Vitro Assembly of the Bam complex

3.1 Machines that Assemble Themselves

The macromolecular machines that are required to assemble cellular components also depend on their own activities for their assembly. For example, the protein components of ribosomes are ribosomally synthesized; the α-helical components of the protein secretion channel are inserted into the membrane by the Sec machine; and the central component of the Bam complex, BamA, is assembled by the Bam complex. Although the ribosome is a ribozyme, 50 years of in vitro and in vivo studies have indicated that the ribosomal proteins are critical for the efficient assembly of functional ribosomes (1, 2). Ribosome assembly proceeds through a series of intermediates in which RNA folding events are influenced by the sequential binding of ribosomal proteins (3-5). The components of the Sec translocon, Sec Y, E, and G, consist of ten, three, and two integral membrane α-helices, respectively; the insertion of these proteins into the inner membrane has not been specifically studied, but it is believed to be mediated by the Sec translocon (6). Similarly, genetic depletion experiments suggest that Bam complexes that are already in the OM mediate the β-barrel assembly of additional molecules of BamA (7). Therefore, *E. coli* cells must contain ribosomes, Sec machines, and Bam complexes in order to make more ribosomes, Sec machines, and Bam complexes.

These cellular machines are universally conserved, and because they each handle many substrates, defects in their assembly can have disastrous effects. Their assembly processes, therefore, must be robust and may be conserved across species. A machine that catalyzes its own assembly also raises questions about how the first such machine was assembled, how it evolved to its current state, and whether its assembly still depends on the more primitive (or conserved)

elements of the machine. Because these machines are essential, it can be difficult to answer these questions with experiments performed in vivo; a cell without any ribosomes, Sec machines, or Bam complex cannot be generated. By studying the assembly process in vitro, it becomes possible to monitor how these complexes are formed *de novo*.

In addition to revealing how the Bam complex assembles, the mechanism of BamA assembly may exemplify the more general β-barrel assembly mechanism. The Bam complex may handle the approximately 100 different OMPs in the OM slightly differently because their structures present different assembly challenges. BamA (or its orthologs), however, must be assembled in all cells that contain integral membrane β-barrels. Although the β-barrel assembly machines in different species differ in their accessory components, they all must be able to assemble a protein that resembles BamA. We hypothesized that all BamA orthologs are assembled by the same mechanism and sought to determine the importance of the Bam proteins in performing this transformation, which occurs in all species.

3.2 The Bam Complex Assembles BamA In Vitro without a Chaperone

Genetically deleting or depleting members of the Bam complex results in lower levels of the abundant OMPs in the OM; on this basis, it is assumed that the Bam complex assembles all β-barrels in the OM, including itself (8, 9). In addition, a BamA mutant lacking POTRA domain 2 was shown to assemble into the OM in the absence of a wild-type copy of BamA, whereas mutants lacking POTRA domains 3, 4, or 5 were not assembled. These experiments suggest that BamA catalyzes its own assembly and that some of the POTRA domains are required in that process (7). However, in vitro experiments are the only direct way to determine whether BamA is a substrate of the Bam complex or whether it uses another mechanism (which does not require

a preassembled Bam complex) to fold and insert itself. The gel-based assay for β-barrel folding described in Chapter 2 was used to examine the ability of the Bam complex to assemble BamA in vitro. These folding reactions were performed in much the same way as those with OmpT: (1) urea-denatured BamA was diluted with or without a chaperone into proteoliposomes containing the Bam complex; (2) the reaction products were separated on semi-native SDS-PAGE; and (3) the folded and unfolded forms of BamA were then visualized. To distinguish newly assembled BamA from the BamA initially in the membrane, a FLAG-tag was added to the N-terminus of the substrate BamA, and it was specifically detected by western blotting with anti-FLAG antibodies. (Many attempts were made to over-express and purify denatured, ^{35}S-labeled BamA, but these were unsuccessful.)

BamA is assembled by the five-protein Bam complex in proteoliposomes (Figure 3.1A). When the substrate is diluted directly into the proteoliposomes or is first preincubated with SurA, it folds in the presence of the Bam complex but not in its absence. If the substrate is preincubated in urea-free buffer or in a solution containing another periplasmic chaperone, Skp, no folding is observed. Therefore, unfolded BamA does become less foldable in the absence of solubilizing factors. SurA can apparently maintain the folding competence of BamA and deliver it to the Bam complex, but even when high SurA concentrations are employed, the yield of folded protein does not substantially exceed that observed when the substrate is diluted directly from urea (Figure 3.1B). The fact that Skp does not support BamA folding may imply that it is not effective in binding unfolded BamA or that, once bound, it cannot release the substrate to the Bam complex. Although Skp has been cross-linked to OMP substrates in vivo, it is not clear whether it delivers them to the Bam complex or simply sequesters them prior to degradation (10-

13). This latter role could explain why it is genetically connected to DegP, the periplasmic OMP protease, in a pathway parallel to that of substrates handled by SurA (14).

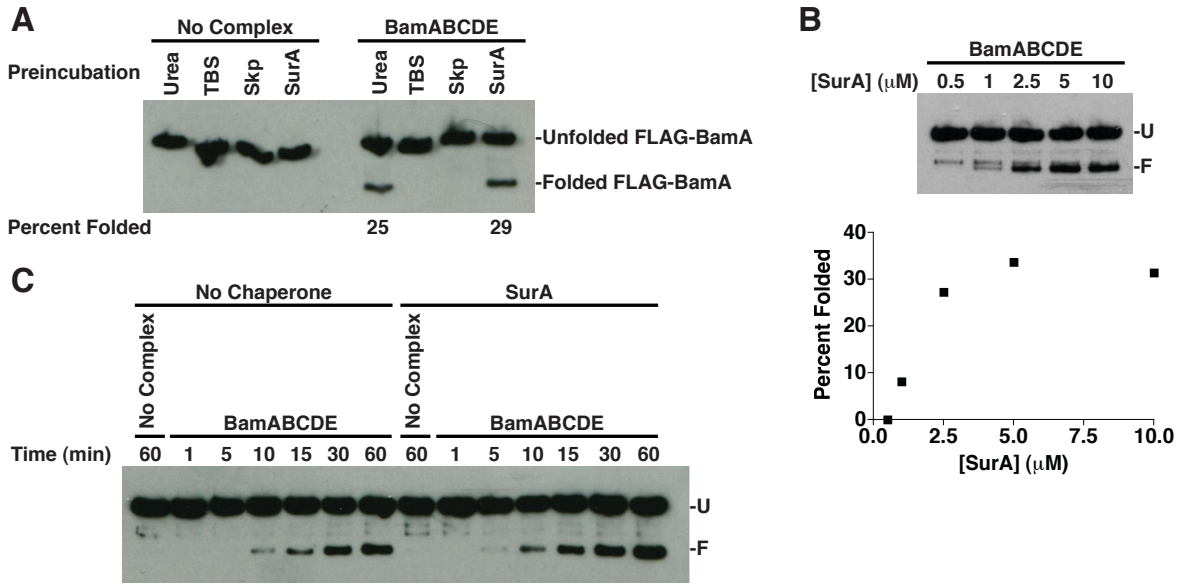

Figure 3.1. BamA can be assembled by the Bam complex without a chaperone. **A.** Urea and SurA maintain the folding competence of the BamA substrate equally well. BamA bearing an N-terminal FLAG-tag was prepared in 8 M urea and then diluted directly into empty liposomes or proteoliposomes containing the Bam complex, or the denatured substrate was first incubated in solutions of tris-buffered saline (TBS), Skp, or SurA and then diluted. The final concentrations of the substrate, Skp, and SurA were 0.5 μM, 15 μM, and 5 μM, respectively. The folding of FLAG-BamA was evaluated after 60 minutes by western blotting with anti-FLAG antibodies. **B.** Large excesses of SurA do not improve the folding yield above that produced when the FLAG-BamA is diluted directly from urea. FLAG-BamA was preincubated with a 1, 2, 5, 10, or 20-fold molar excess of SurA and then added to the BamABCDE proteoliposomes. The folding yield reaches a maximum at a final SurA concentration of 5 μM, which is the same concentration used in **A**. (U: unfolded FLAG-BamA, F: folded FLAG-BamA) **C.** SurA does not affect the kinetics of BamA assembly by the Bam complex. FLAG-BamA was diluted directly from urea or preincubated in SurA and then diluted into proteoliposomes. The folding reactions were stopped at the indicated time points.

Although SurA does not improve the final yield of folded BamA, the time scales for BamA folding following direct addition from a urea solution or after a preincubation with SurA were compared in order to determine whether the chaperone changes the pathway of BamA assembly such that its kinetics improve (Figure 3.1C). BamA folds over the course of 60 minutes at roughly the same rate when SurA is present or absent. Therefore, SurA is able to maintain unfolded BamA in a folding-competent state, but it is not specifically required to deliver it to the Bam complex—it can be replaced by a high concentration of urea. The apparent

longer time scale of BamA folding compared to that of OmpT, which was complete after approximately ten minutes, may imply that BamA is less efficiently transferred to or handled by the Bam complex or that it remains soluble or foldable for a longer period of time.

3.3 The POTRA Domains of BamA Affect the Foldability of its β-Barrel

Perhaps the chaperone-independence and longer lifetime of the BamA substrate reflect the fact that it contains five soluble POTRA domains. Truncated BamA substrates with decreasing numbers of POTRA domains were generated, and the ability of the Bam complex to assemble these constructs was determined. The truncations do not appear to affect the stability of the β-barrel substantially; all of the substrates can fold to form a heat-modifiable β-barrel in detergent (Figure 3.2A). However, the Bam complex does not fold those lacking POTRA domains 4 and 5; POTRA domains 1-3 can be deleted without any significant detriment to the folding, but P4 and P5 cannot (Figure 3.2B). The POTRA domains may affect the folding-competence of BamA nonspecifically—simply by increasing its solubility to prevent it from aggregating before it interacts with the Bam complex. If the failure of the BamAΔP1-4 and BamAΔP1-5 substrates to fold is due only to their insolubility, SurA might be expected to ameliorate their aggregation and make them more suitable for assembly. This is not the case; incubating the BamAΔP1-4 and BamAΔP1-5 substrates with SurA does not result in any folding of their β-barrels (Figure 3.2C). POTRA domains 4 and 5, therefore, may be involved in the β-barrel assembly in a more direct, or specific manner.

The POTRA domains are on the N-terminus of BamA and consequently emerge from the Sec machine before the β-barrel. The structures of the POTRA domains indicate that they do not form extensive contacts with each other and thus may be able to fold independently (7, 15, 16).

Perhaps the POTRA domains fold as they are secreted into the periplasm and then bind the unfolded β-barrel of BamA in the periplasm before it is delivered to the Bam complex in the OM. They might specifically interact with the β-strands of the incipient β-barrel of BamA by β-augmentation (7, 15, 17). This intramolecular chaperone mechanism could explain the slower folding of the BamA substrate. The POTRA domains in the Bam complex effectively must compete with those in the BamA substrate for binding to the unfolded β-barrel.

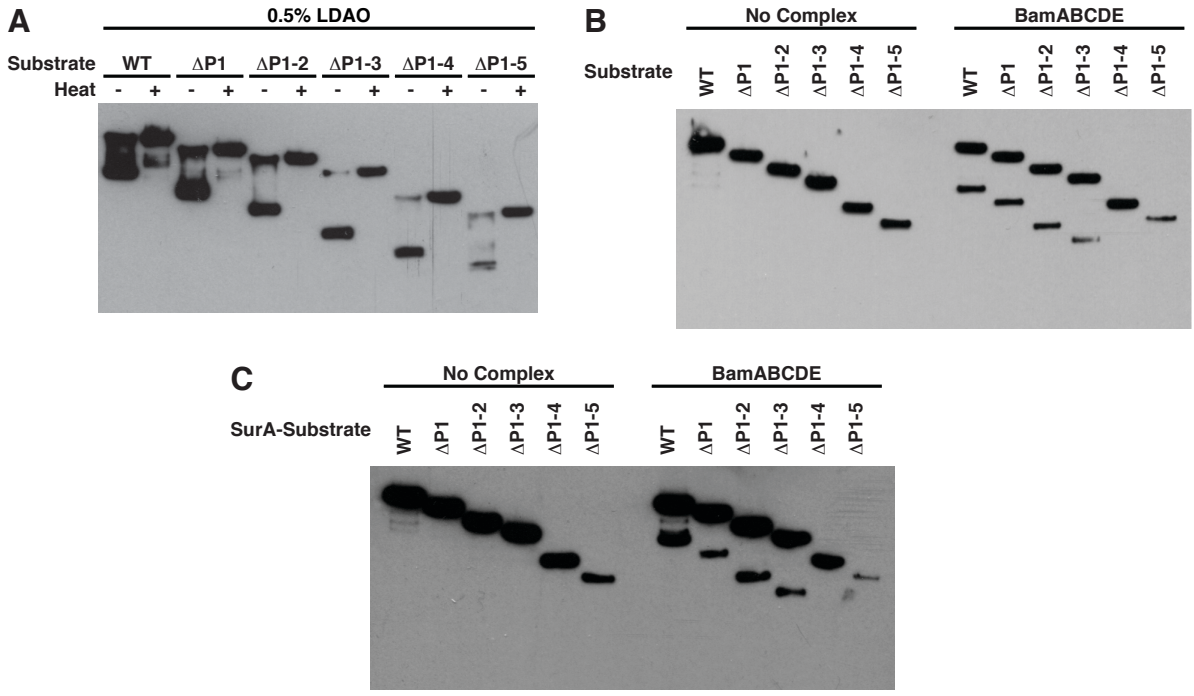

Figure 3.2. BamA substrates lacking four or five POTRA domains cannot be folded by the Bam complex. **A.** None of the POTRA domains are required for the β-barrel of BamA to fold in detergent. Denatured FLAG-BamA substrates with decreasing numbers of POTRA domains were diluted into 0.5% LDAO. Folding was stopped after 60 minutes and the samples were run on semi-native SDS-PAGE with and without prior boiling. The bands of lower apparent molecular weight in the unheated samples contain a folded β-barrel. **B.** POTRA domains 1, 2, and 3 are not required for the assembly of BamA by the Bam complex. The truncated BamA substrates were diluted from urea into empty liposomes or proteoliposomes containing the Bam complex, and folding was stopped after 60 minutes. **C.** SurA does not change the folding competence of the truncated BamA substrates. The experiment was performed as in **B** except that the substrates were preincubated with a ten-fold molar excess of SurA prior to their addition to the liposomes or proteoliposomes.

It remains an open question whether any of the POTRA domains have specifically evolved to chaperone the β-barrel of BamA. The Bam complex can assemble BamA mutants bearing individual deletions of any of the first four POTRA domains in the cell (7). (A mutant

lacking P5 displays toxicity depending on the strain background and whether it bears an N-terminal histidine tag.) These results imply that no single POTRA domain is specifically required for the folding-competence of the β-barrel, but it is difficult to evaluate how juxtaposing POTRA domains that are not normally adjacent to one another affects their function and how these various mutants might rely on periplasmic chaperones. As shown here, truncating the POTRA domains and evaluating their folding in vitro makes it apparent that two POTRA domains are minimally required for the folding competence of the BamA β-barrel. The requirement for P4 and P5 is consistent with the in vivo essentiality of these domains, but they could have other essential functions and it is not yet known whether any two POTRA domains would suffice to chaperone BamA to the OM (7). Additional mutational studies that would replace P4 and P5 with other POTRA domains or that would limit the ability of these domains to bind peptides by β-strand augmentation could clarify how they affect the assembly of the attached β-barrel.

3.4 The Conserved Components of the Bam Complex Assemble BamA

The POTRA domains in the BamA substrate play a role in its assembly, but they are not sufficient; the Bam complex facilitates the overall process. The Bam subcomplexes were used to determine whether the entire complex is required for efficient assembly of BamA (as is the case with OmpT) or whether some of the lipoprotein components are dispensable. The BamAB and BamACDE subcomplexes display similar activity to the complete complex in folding BamA (Figure 3.3A). No specific lipoprotein (including the essential protein, BamD) appears to be required, and BamA may be solely, or primarily, responsible for assembling additional

molecules of BamA. In other words, BamA does not assemble itself efficiently into an empty liposome, but any complex containing BamA facilitates the assembly of more BamA.

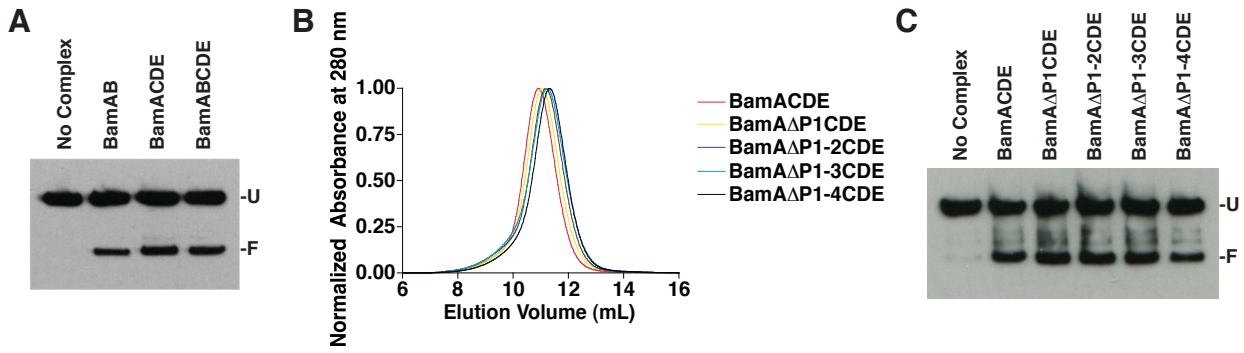

Figure 3.3. The Bam lipoproteins and first four POTRA domains of BamA are not required in the membrane to assemble additional molecules of BamA. **A.** Bam subcomplexes lacking the lipoproteins demonstrate activity equal to that of the complete complex in assembling full-length FLAG-BamA. **B.** Size exclusion chromatograms of the truncated BamACDE complexes suggest that they have similar stability. **C.** BamACDE subcomplexes bearing truncations of the POTRA domains all assemble full-length FLAG-BamA.

The POTRA domains are clearly important in the BamA substrate. To determine whether they are also required in the OM to assemble other BamA molecules, BamACDE complexes containing truncations of BamA were over-expressed, purified, and incorporated into proteoliposomes. These complexes all have similar stability as judged by their behavior on size exclusion chromatography and demonstrate similar activity in folding full-length BamA (Figures 3.3B and C). Therefore, POTRA domains 1-4 are not required to assemble other molecules of BamA. A complex lacking all five POTRA domains cannot be purified because BamC, D, and E bind to the fifth POTRA domain, and the isolated β-barrel of BamA cannot be purified to homogeneity after cytoplasmic expression and in vitro folding in detergent. Consequently, the importance of P5 cannot be directly evaluated, but these results suggest that the minimal pieces of the Bam complex required to assemble BamA are the β-barrel and fifth POTRA domain. The smallest homologs of BamA, the mitochondrial Sam50 (or Tob55) proteins, contain only these two domains. Furthermore, the BamA ortholog from *Neisseria meningitidis* has been shown to

assemble in yeast mitochondria, which suggests that the one POTRA domain in the Sam complex is sufficient to assemble this protein in vivo and that the mechanisms by which the Sam and Bam complexes function are sufficiently similar to handle each other's substrates (18, 19). As the β-barrel assembly complexes in bacteria and mitochondria have evolved apart from each other, the ability to make more of themselves has been retained within the same domains.

3.5 BamA Assembles into an Active Complex In Vitro

The experiments described thus far have indicated that one molecule of BamA lacking the first four POTRA domains may be sufficient to facilitate the assembly of additional BamA molecules and hence that BamA assembly could increase exponentially as newly assembled BamA molecules become active and start assembling more BamA. The assembly of BamA into proteoliposomes containing different Bam complex components was monitored over time (Figure 3.4). Compared to the activity of the BamACDE subcomplex, BamA does not fold BamA efficiently on its own (compare lanes 2-7 to lanes 14-19 in Figure 3.4A, and the blue and black curves in Figure 3.4B). Therefore, although none of the Bam lipoproteins seems to be specifically required to fold BamA, these proteins do affect the activity of BamA. The BamAB and BamACDE subcomplexes can both fold BamA, but removing all of the lipoproteins produces a much less active machine.

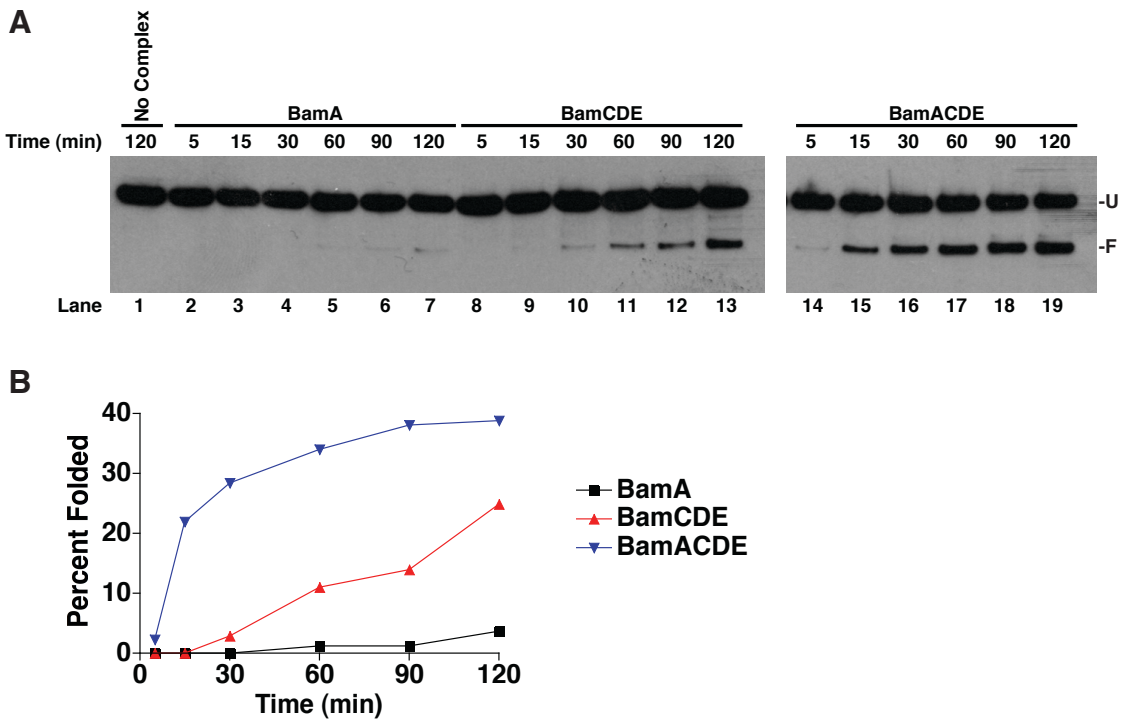

Figure 3.4. The rate of BamA assembly increases in proteoliposomes containing the BamCDE lipoproteins. **A.** Western blot of BamA assembly into proteoliposomes containing, BamA, BamCDE, BamACDE over the course of two hours. Reactions were stopped at the indicated time points and run on semi-native SDS-PAGE without prior boiling. **B.** Plot of the percentage of folded FLAG-BamA in each reaction in **A** over time.

Surprisingly, the rate of assembly of BamA into proteoliposomes containing only the BamC, D, and E lipoproteins appears to increase with time (Figure 3.4A, lanes 8-13, and Figure 3.4B, red curve). Based on the experiments described in the section 3.4, vesicles that do not contain any BamA would not be expected to assemble BamA more effectively than those that do. These observations can be reconciled if a small amount of BamA assembles into a complex with the BamCDE lipoproteins, which would then have higher activity and facilitate more BamA assembly. This model requires an initial BamA folding and insertion event to occur in the absence of any preassembled BamA, and it could thereby explain why a lag phase is observed in the BamCDE reaction and not in the BamACDE reaction. OMPs, including BamA, have been shown to spontaneously assemble into lipid vesicles in the absence of the Bam complex, and the efficiency of this process depends on the particular lipids used (20). The activity of the

BamCDE proteoliposomes does vary from experiment to experiment, which may reflect the stochastic nature of the first assembly event. The length of the initial lag phase will depend on when the spontaneous insertion of BamA occurs and how long it takes for it to associate with the lipoproteins.

This model also implies that the Bam lipoproteins alter the mechanism of BamA assembly either directly or indirectly. They may stabilize a more active conformation of BamA in the membrane or assist in the assembly steps it catalyzes (21). Additional experiments will be required to verify the formation of a BamACDE complex in these reactions, but the increase in folding activity over time implies that BamA is assembled into an active state in these proteoliposomes.

3.6 Conclusion

The Bam complex contains five components, but they are not all required to assemble all β-barrels. In fact, the accessory proteins associated with the orthologs of BamA in other Gram-negative bacteria and in the mitochondria of eukaryotes can vary widely. For example, although the β-barrels of the *E. coli* and mitochondrial β-barrel assembly machines are oriented in the same direction (*i.e.* with the POTRA domains in the compartment between the inner and outer membranes), the *E. coli* machine contains four lipoproteins on the periplasmic face of the OM and the mitochondrial complex contains two peripheral membrane proteins on the cytoplasmic (or outer) surface of the outer mitochondrial membrane (Figure 3.5). Therefore, the accessory proteins likely contribute to the assembly mechanism in different ways in different species and have evolved to assemble the particular β-barrels in those membranes or to coordinate β-barrel assembly with other processes. The experiments described in this chapter directly demonstrate

that the components required to assemble the one universally present β-barrel, BamA, are the components that are universally conserved. The β-barrel and adjacent POTRA domain of all BamA orthologs are likely responsible for performing the steps in the β-barrel assembly mechanism that are the same in all species.

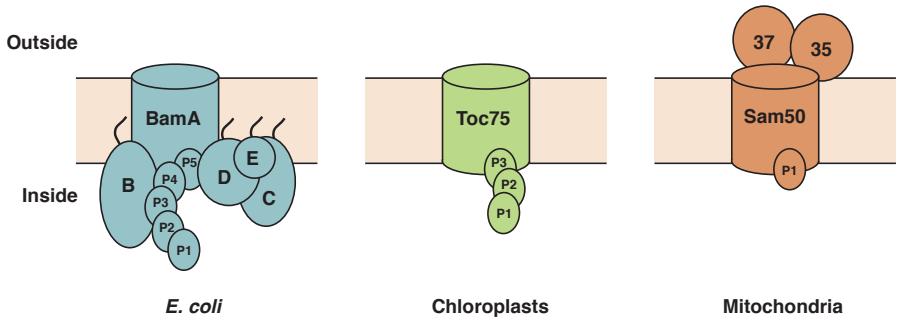

Figure 3.5. The β-barrel assembly machines in *E. coli*, chloroplasts, and mitochondria. These machines contain different accessory components and different numbers of POTRA domains (labeled P1-5). All Gram-negative BamA homologs contain five POTRA domains; all chloroplast homologs contain three; and all mitochondrial homologs contain one. The β-barrel and its adjacent POTRA domain are the only components found in all organisms that assemble β-barrels.

However, other components of the Bam complex have been found to be essential in *E. coli*; individual deletions of BamD, or of POTRA domain 3, 4, or 5 lead to cell death. P4 may be required to chaperone BamA to the membrane, and P5 may share this function and also be required to assemble additional molecules of BamA once it is in the membrane, but the essentiality of P3 and BamD cannot be explained by the results presented here. It is possible that BamD and P3 affect the assembly of a group of OMPs that are not individually essential but are in combination, or that BamD and P3 are specifically required to assemble LptD, the only other known essential OMP. LptD forms a complex with an essential lipoprotein, LptE, in which the lipoprotein is believed to reside inside the β-barrel of LptD. The formation of this unusual two-protein plug and barrel architecture and of a set of non-consecutive disulfide bonds makes the assembly of LptD significantly more complex (22-26). It would not then be surprising if additional Bam components were required to coordinate its assembly. In fact, mutations in LptD

that impair its assembly make the OM leaky, and mutations that suppress this phenotype have been found in the third POTRA domain of BamA and in BamB, which binds to P3 (27-29). The *lptD4213* allele, which produces the leaky phenotype, is lethal in combination with a *bamD* deletion when the cells are incubated at 37 ^{o}C (9). BamB, BamD, and P3 are thus genetically linked to LptD, but additional experiments will be required to determine if and how they directly affect LptD assembly.

The assembly of an active Bam complex in vitro demonstrates how a low efficiency machine can become a high one. BamA alone can assemble more BamA at a very slow rate, but when the lipoproteins are present, a very small amount of folded BamA can form a more active complex that can then rapidly amplify the amount of folded BamA. This phenomenon provides a mechanism for rapidly restoring OMP assembly if the BamA population was ever depleted or damaged in the cell by environmental stress. If BamA assembly absolutely required preexisting Bam complexes, cells in this situation would be unable to recover. If a small amount of BamA can chaperone itself to and fold itself in the OM, the cell might be able to survive long enough to assemble a Bam complex, which could then rapidly increase the number of Bam complexes.

The assembly of BamA also indicates that aspects of the β-barrel assembly mechanism in *E. coli* are the same in all organisms while others reflect the particular requirements of the membrane in which it occurs. Although BamA only requires the conserved components of the Bam complex to assemble, the lipoproteins dramatically increase their activity. The β-barrel and fifth POTRA domain of BamA perform the general chemistry required to assemble a β-barrel, and the lipoproteins adapt that chemistry to assemble the approximately 100 different OMPs in the *E. coli* OM. Perhaps the BamA ancestor, from which the orthologs in Gram-negative bacteria, mitochondria, and chloroplasts evolved, could assemble itself efficiently unaided;

however, as the accessory proteins evolved to fold the substrates needed for these organisms and organelles to thrive in their specific environments, the BamA orthologs became more reliant on them for their own assembly. Remarkably, the in vitro assembly of BamA reflects this evolutionary history.

3.7 Materials and Methods

3.7.1 Materials

All vectors used for plasmid construction were obtained from Novagen, and restriction enzymes were purchased from New England BioLabs. Unless noted otherwise, all cultures were grown in LB media from Difco with the appropriate antibiotics added at concentrations of 50 μg/mL. The n-dodecyl-β-D-maltopyranoside (DDM) and lauryldimethylamine-N-oxide (LDAO) used in the Bam complex purifications were purchased from Anatrace, and the tris(2-carboxyethyl)phosphine (TCEP) was purchased from Hampton Research. The Ni-NTA and TALON resins were purchased from Qiagen and Clontech, respectively. All size exclusion chromatography steps were performed using a Superdex 200 column from GE Healthcare. The *E. coli* phospholipids used in preparing the proteoliposomes were obtained from Avanti Polar Lipids as a dried polar lipid extract. All protein concentrations were determined using the Bio-Rad DC Protein Assay. The folded and unfolded forms of the FLAG-BamA substrates were detected on western blots with monoclonal anti-FLAG M2-peroxidase (HRP) mouse antibody from Sigma-Aldrich and Lumigen PS-3 detection reagent from GE Healthcare.

3.7.2 Plasmid Construction

The plasmids used for over-expression of the proteins in this study are described in Table 3.1. All genes were amplified from MC4100 chromosomal DNA using the primers listed in Table 3.2. The restriction sites highlighted in bold were used to construct the indicated plasmids. The sequences that were mutated or inserted by site directed mutagenesis are also highlighted in bold in the appropriate primers.

3.7.3 Expression and Purification of the Bam Complex and Subcomplexes

3.7.3.1 BamABCDE and BamAB

The five-protein Bam complex and the BamAB subcomplex were expressed and purified as described in Chapter 2 with the following exceptions: (1) BamA and BamB were expressed in BL21(DE3) strains carrying pSK38 or pSK86 grown in 2xYT media from Difco, and (2) the His_6-tag on the N-terminus of BamA was not removed by thrombin digestion in the BamAB purification. The BamA and BamB proteins were found to express at higher levels in 2xYT media compared to LB, and the His-tag removal step was omitted to increase the yield of the BamAB complex.

3.7.3.2 BamACDE and Truncated BamACDE

BamA and BamA variants containing serial POTRA deletions, BamAΔP1, BamAΔP1-2, BamAΔP1-3, and BamAΔP1-4, were expressed as inclusion bodies in BL21(DE3) strains transformed with pCH103, pSK131, pCH36, pSK133, and pSK134, respectively. Cultures of these strains were grown at 37 ^{o}C to OD_{600} = 0.4. Expression of the BamA proteins was then induced by addition of 0.1 mM IPTG, and the cultures were incubated for another 3-4 hours. The cells were then harvested, resuspended in TBS (pH 8) (*i.e.* 20 mM Tris-HCl (pH 8), 150 mM NaCl), and lysed by French press. They were centrifuged at 5,000 x g for 10 min at 4 ^{o}C, and the pellets were resuspended in 8 M urea. After rocking at room temperature for about an hour, the solutions were centrifuged at 18,000 x g for 10 min at 4 ^{o}C. The supernatants contained the denatured BamA variants. These urea solutions were diluted ten-fold into TBS (pH 8), 0.5% LDAO and incubated on a rocker at 4 °C overnight to allow the β-barrels of these BamA variants to fold. The solutions were then centrifuged at 5,000 x g for 10 minutes to remove aggregated

material. The three lipoproteins, BamCDE, were expressed as described in the reconstruction of the five-protein complex in Chapter 2, and the solubilized membranes were mixed with the refolded BamA variants. The four-protein complexes were then purified by Ni-NTA affinity chromatography (using the His-tag on BamE) in TBS (pH 8), 0.05% DDM. Finally, the eluates were concentrated and subjected to size exclusion chromatography in TBS (pH 8), 0.03% DDM, 1 mM TCEP.

3.7.3.3 BamCDE

As described in the purification of the five-protein complex in Chapter 2, BamC, BamD, and BamE were coexpressed in a BL21(DE3) strain carrying both pSK46 and pBamE-His. The cells were harvested, lysed, and the membrane fraction was isolated, solubilized, and dialyzed overnight as described in Chapter 2. The BamCDE complex was then directly purified from the solubilized membranes by Ni-NTA affinity chromatography in TBS (pH8), 0.05% DDM. The eluate was concentrated and subjected to size exclusion chromatography in TBS (pH 8), 0.03% DDM, 1 mM TCEP.

3.7.3.4 BamA

BamA was expressed in a BL21(DE3) strain carrying pCH103, which encodes the protein without its first 22 amino acids. The protein is thus expressed in the cytoplasm without its signal sequence. Cultures of this strain were grown at 37 ^{o}C to OD_{600} = 0.4. The protein expression was then induced by addition of 0.1 mM IPTG, and the cultures were incubated for another 2-3 hours. The cells were then harvested, resuspended in TBS (pH 8), and lysed by French press. They were then centrifuged at 5,000 x g for 10 min at 4 ^{o}C to pellet the BamA

inclusion bodies. These inclusion bodies were dissolved in 8 M urea by incubation with rocking at room temperature for approximately an hour. This solution was centrifuged at 18,000 x g for 10 min at 4 ^{o}C to pellet any undissolved material. The supernatant was then diluted ten-fold into a stirred solution of TBS (pH 8), 0.5% LDAO by dropwise addition of the denatured protein. This solution was incubated overnight at 4 ^{o}C to allow the β-barrel of BamA to fold. It was then concentrated and subjected to size exclusion chromatography in TBS, 0.03% DDM, 1 mM TCEP to exchange the detergent and to separate the folded BamA from the remaining unfolded material. Samples from each fraction were run on semi-native SDS-PAGE to identify those containing the folded BamA; these were then concentrated and subjected to a second round of size exclusion chromatography in TBS (pH 8), 0.03% DDM, 1 mM TCEP to remove any residual unfolded BamA.

3.7.4 Expression and Purification of Chaperone Proteins

His_6-SurA was expressed and purified as described in Chapter 2. Skp-His_6 was expressed in a BL21(DE3) strain carrying pCH24, which encodes the protein without its signal sequence such that it is expressed in the cytoplasm. Skp-His_6 was expressed and purified according to the same methods used for His_6-SurA.

3.7.5 Expression and Purification of FLAG-BamA and Truncated FLAG-BamA Substrates

BamA and BamA variants containing serial POTRA truncations (BamAΔP1 – BamAΔP1-5) were expressed in the cytoplasm with N-terminal FLAG-tags in BL21(DE3) strains carrying pCH128 – pCH133, respectively. Cultures of these strains were grown at 37 ^{o}C

to OD_{600} = 0.4. Expression of the proteins was then induced by addition of 0.1 mM IPTG, and the cultures were incubated for another 2-3 hours. The cells were then harvested, resuspended in TBS (pH 8), and lysed by French press. They were then centrifuged at 5,000 x g for 10 min at 4 ^{o}C to pellet the inclusion bodies containing the BamA proteins. These inclusion bodies were dissolved in 8 M urea by incubation with rocking at room temperature for approximately an hour. The solutions were then centrifuged at 18,000 x g for 10 min at 4 ^{o}C to pellet any undissolved material. These clarified urea solutions contained only minor amounts of other contaminating proteins as judged by SDS-PAGE, so the denatured BamA proteins were used in folding assays without further purification.

3.7.6 Proteoliposome Preparation

Proteoliposomes containing the Bam complex and Bam subcomplexes were prepared by the detergent dilution methods described in Chapter 2. Briefly, *E. coli* phospholipids (40 μL of a 20 mg/mL sonicated aqueous suspension) were added to the purified Bam complexes (200 μL of 10 μM solutions) in TBS (pH 8), 0.03% DDM, 1 mM TCEP and incubated on ice for five minutes. These phospholipid, detergent, protein complex mixtures were then diluted with 8 mL of TBS (pH 8), incubated on ice for 30 minutes, and then ultracentrifuged at 300,000 x g for 2 hours at 4 ^{o}C. The pelleted proteoliposomes were then resuspended in 200 μL of TBS (pH 8). Any proteoliposomes that were not used immediately were flash frozen in liquid nitrogen and stored at -80 °C.

3.7.7 FLAG-BamA Folding Assay

3.7.7.1 FLAG-BamA Folding into Bam Proteoliposomes

FLAG-BamA was prepared at a concentration of 5 μM in 8 M urea and then diluted ten-fold into solutions containing empty liposomes or the Bam proteoliposomes. The proteoliposomes were also diluted four-fold from their stock concentrations in these reactions. A typical reaction contained 2.5 μL liposomes or proteoliposomes, 6.5 μL TBS (pH 8), and 1 μL of 5 μM FLAG-BamA substrate such that the final concentrations of the substrate and Bam complex were 0.5 μM and ~2.5 μM, respectively. If a chaperone was included in the reaction, it was preincubated with the FLAG-BamA substrate for 10 minutes, and then this chaperone-BamA mixture was diluted ten-fold into the proteoliposomes. Specifically, the FLAG-BamA substrate was diluted ten-fold from a 50 μM solution in 8 M urea into a solution of the chaperone in TBS (pH 8). Unless noted otherwise in the figures, the concentrations of Skp-His_6, His_6-SurA, and the BamA substrates were, respectively, 150 μM, 50 μM, and 5 μM in these preincubation solutions, and 15 μM, 5 μM, and 0.5 μM in the final reactions. After 60 minutes of incubation of the substrate with the proteoliposomes at room temperature, the reactions were stopped by adding 2% SDS sample loading buffer. (For the experiments in Figure 3.1C and Figure 3.4, aliquots of the reactions were removed at the indicated time points and stopped by the same method.) One-tenth of the reaction volume was applied to SDS-PAGE (4-20% gradient gel), and run at 150 V for 110 minutes at 4 ^{o}C. The proteins were transferred from the gel to a PVDF membrane by semi-dry transfer in 25 mM Tris-HCl, 192 mM glycine (pH 8.3) at 10 V for one hour. The products of the reaction were detected by western blotting with FLAG-HRP antibodies (used at a dilution of 1:200,000). The western blots were scanned, and ImageQuant TL was used to calculate the densities of the observed bands. The percent yields of folded

FLAG-BamA were determined by comparing the densities of the unfolded and folded bands in each lane.

3.7.7.2 FLAG-BamA Folding in Detergent

In the experiment in Figure 3.2A, the full-length and truncated FLAG-BamA substrates were prepared at a concentration of 5 μM in 8 M urea. They were then diluted ten-fold into a solution of TBS (pH 8), 0.5% LDAO and incubated at room temperature for one hour. The folding reactions were then stopped with 2% SDS sample loading buffer. One-tenth of each reaction was run on semi-native SDS-PAGE, and the products were detected by western blotting as described in the previous section.

Table 3.1 Plasmids used in this study

Name	Description	Construction
pCH103	pET22b-bamA (E22-W810)	PCR with primers: bamA(22-810)-5' and bamA(22-810)-3' and then site directed mutagenesis with primers: bamA-stop-5' and bamA-stop-3'
pSK131	pET22b-bamAΔP1 (E90-W810)	PCR with primers: p2N2 and bamA-Ce
pCH36	pET22b- bamAΔP1-2 (G172-W810)	PCR with primers: p3N2 and bamA-Ce and then site directed mutagenesis with bamA-G172-5' and bamA-G172-3'
pSK133	pET22b-bamAΔP1-3 (D264-W810)	PCR with primers: p4N2 and bamA-Ce
pSK134	pET22b-bamAΔP1-4 (G344-W810)	PCR with primers: p5N2 and bamA-Ce
pSK135	pET22b-bamAΔP1-5 (N422-W810)	PCR with primers: CtN2 and bamA-Ce
pCH128	pET22b-FLAG-bamA (A21-W810)	Site directed mutagenesis in pCH103 with primers: FLAG-nsbamA-for and FLAG-nsbamA-rev
pCH129	pET22b-FLAG-bamAΔP1 (E90-W810)	Site directed mutagenesis in pSK131 with primers: FLAG-ΔP1-for and FLAG-ΔP1-rev
pCH130	pET22b-FLAG-bamAΔP1-2 (G172-W810)	Site directed mutagenesis in pCH36 with primers: FLAG-ΔP1-2-for and FLAG-ΔP1-2-rev
pCH131	pET22b-FLAG-bamAΔP1-3 (D264-W810)	Site directed mutagenesis in pSK133 with primers: FLAG-ΔP1-3-for and FLAG-ΔP1-3-rev
pCH132	pET22b-FLAG-bamAΔP1-4 (G344-W810)	Site directed mutagenesis in pSK134 with primers: FLAG-ΔP1-4-for and FLAG-ΔP1-4-rev
pCH133	pET22b-FLAG-bamAΔP1-5 (N422-W810)	Site directed mutagenesis in pSK135 with primers: FLAG-ΔP1-5-for and FLAG-ΔP1-5-rev
pCH24	pET22b-Skp-His_6 (A21-K161)	PCR with primers: Skp-Nns and Skp-CHis

Table 3.2 Primers used in plasmid construction

Name	Sequence
bamA(22-810)-5’	ACCA**CATATG**GAAGGGTTCGTAGTGAAAGATATTCATTTCGA
bamA(22-810)-3’	ATTA**GCGGCCGC**CCAGGTTTTACCGATGTTAAA
bamA-stop-5’	AACATCGGTAAAACCTGG**TAA**GCGGCCG
bamA-stop-3’	GTGCTCGAGTGCGGCCGC**TTA**CCAGGT
p2N2	AGAG**CATATG**GAACGTCCGACCATTGCCAGC
p3N2	AGAG**CATATG**GTGTCAGCTGAAATCCAGCAAATTAAC
p4N2	AGAG**CATATG**GATCAGTACAAGCTTTCTGGCGTTG
p5N2	AGAG**CATATG**GGTAACCGTTTCTACGTGCGTAAG
CtN2	AGAG**CATATG**AACACCGGTAGCTTCAACTTTGG
bamA-Ce	ACAC**GCGGCCGC**TTACCAGGTTTTACCGATGTTAAACTG
bamA-G172-5’	TATACATATG**GGT**GTGTCAGCTGAAATCCAGCAAATT
bamA-G172-3’	GCTGACAC**ACC**CATATGTATATCTCCTTCTTAAAGTT
FLAG-nsbamA-for	CATATG**GACTACAAAGACGATGACGACAAG**GCTGAAGGGTTC GTAGTG
FLAG-nsbamA-rev	CAG C**CTTGTCGTCATCGTCTTTGTAGTC**CATATGTATATCTCCTTCTT
FLAG-ΔP1-for	CATATG**GACTACAAAGACGATGACGACAAG**GAACGTCCGACCATTGCC
FLAG-ΔP1-rev	CGT TC**CTTGTCGTCATCGTCTTTGTAGTC**CATATGTATATCTCCTTCTT
FLAG-ΔP1-2-for	CATATG**GACTACAAAGACGATGACGACAAG**GGTGTGTCAGCTGAAATC
FLAG-ΔP1-2-rev	CACC**CTTGTCGTCATCGTCTTTGTAGTC**CATATGTATATCTCCTTCTT
FLAG-ΔP1-3-for	CATATG**GACTACAAAGACGATGACGACAAG**GATCAGTACAAGCTTTCT
FLAG-ΔP1-3-rev	GATC**CTTGTCGTCATCGTCTTTGTAGTC**CATATGTATATCTCCTTCTT
FLAG-ΔP1-4-for	CATATG**GACTACAAAGACGATGACGACAAG**GGTAACCGTTTCTACGTG
FLAG-ΔP1-4-rev	TACC**CTTGTCGTCATCGTCTTTGTAGTC**CATATGTATATCTCCTTCTT
FLAG-ΔP1-5-for	CATATG**GACTACAAAGACGATGACGACAAG**AACACCGGTAGCTTCAAC
FLAG-ΔP1-5-rev	TGTT**CTTGTCGTCATCGTCTTTGTAGTC**CATATGTATATCTCCTTCTT
Skp-Nns	ATGA**CATATG**GCTGACAAAATTGCAATCG
Skp-CHis	ATGA**GCGGCCGC**TTTAACCTGTTTCAGTACG

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Chapter 4: Crystallization of the Bam Complex for Structural Studies

4.1 Mechanistic Hypotheses from Structures of the Periplasmic Components of the Bam Complex

An understanding of the molecular mechanism of the Bam complex will require detailed information about its structure. Structural studies have thus far focused on the soluble domains of the complex—that is, the POTRA domains of BamA and delipidated versions of the lipoproteins. Some of these structures have led to hypotheses about how the Bam complex functions, but a cohesive model of the β-barrel assembly process is still far from clear.

The POTRA domains of BamA have been suggested to bind OMP substrates by β-strand augmentation on the basis of crystal packing interactions observed in two separate structures and on NMR studies using model peptides (1-3). This mechanism is attractive and has been widely accepted because it is specific for a secondary structural element, not a particular amino acid sequence, and because other proteins that bind OMPs use this recognition mechanism. DegS, a periplasmic protease that senses the accumulation of unfolded OMPs and activates the envelope stress response, binds the C-termini of unfolded OMPs by β-strand augmentation (4-6). The POTRA domains might initiate the formation of β-strands by binding unfolded OMPs in this manner, but it would be useful to know whether the binding is dynamic or causes conformational changes in the POTRA domains which might assemble the β-strands into β-hairpins or ultimately a β-barrel. More information is required to determine if β-strand augmentation is simply a way for the Bam complex to distinguish its substrates from other periplasmic proteins, or whether it induces tertiary structure in the substrate. Clearly, not all of the POTRA domains

are required to assemble all OMPs, so any mechanism that invokes β-strand augmentation would have to account for the non-essentiality of some of the POTRA domains (1).

The structures of the lipoproteins have raised similar questions (Figure 4.1). BamB is an eight-bladed β-propellar; it has been suggested that the blades of the propellar could also bind peptides by β-strand augmentation (7-10). A group of residues in BamB that have been shown to cross-link to BamA are all located in a patch on one side of the propellar (11). Perhaps this side of BamB associates with the POTRA domains of BamA and the edges of the propellar aid in organizing the β-strands of substrate β-barrels.

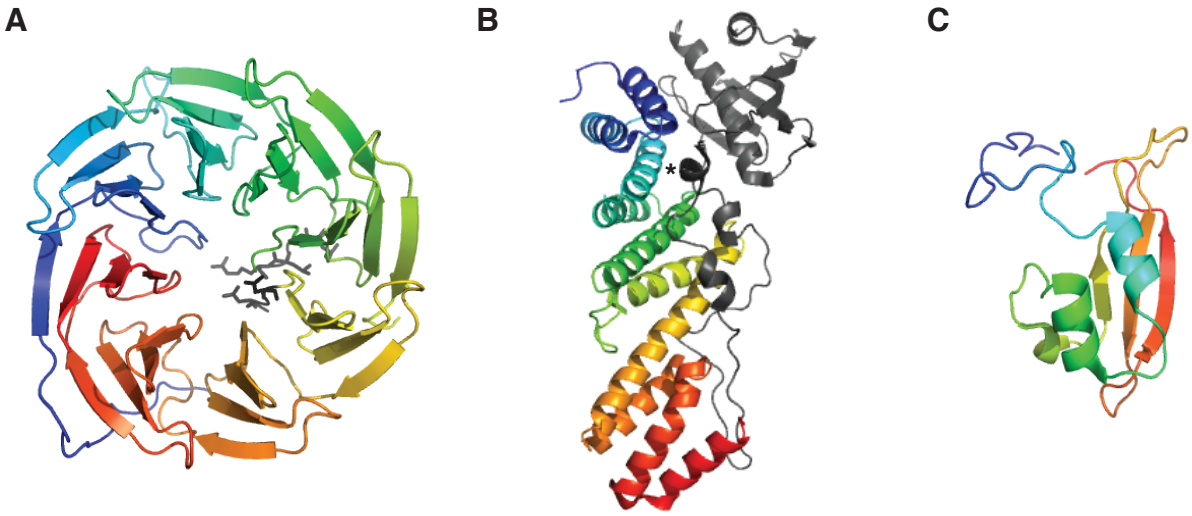


Figure 4.1. Structures of the Bam lipoproteins. **A.** β-propellar structure of BamB (pdb: 3prw). The protein is colored from blue to red from the N-terminus to the C-terminus. The residues on the lower face of the propellar that were shown to cross-link to BamA are shown as black sticks. **B.** The structure of BamD in complex with the N-terminal domains of BamC (pdb: 3tgo). BamD is colored from the N-terminus to the C-terminus from blue to red, and the long loop and first globular domain of BamC are shown in gray. The pocket formed by the first three tetratricopeptide repeats, which is proposed to bind the C-termini of OMP substrates, is indicated with a star. In this structure, BamC partially occludes this pocket. **C.** NMR structure of BamE in solution (pdb: 2kxx). BamE consists of two α-helices packed against a three-stranded β-sheet.

BamC contains two globular domains and an extended loop that binds to BamD along its length; its structure is otherwise uninformative (10, 12). BamD consists of ten α-helices that form five tetratricopeptide repeats (TPRs) (10, 12, 13). TPRs are often involved in protein-

protein interactions, and the first three in BamD form a pocket, which has been proposed to bind the C-terminus of OMP substrates. A C-terminal histidine-tag in one of the BamD crystal structures bound in this pocket, and the pocket is similar to that in other proteins that bind C-terminal peptides (10, 13). Peptides containing the C-terminal sequences of OMPs have been shown to change the channel conductance properties of BamA in vitro and deletions of the C-terminal sequences of mitochondrial OMPs have been shown to impair their assembly (14, 15). The entropic barrier to bringing the N- and C-termini of a β-barrel together is likely one of the major hurdles to OMP assembly; therefore, it is appealing to imagine that the Bam complex may facilitate the process by binding both termini—the N-terminus by BamA by β-strand augmentation and the C-terminus by the TPRs of BamD. This mechanism could thereby explain the essentiality of BamD, but, again, there is no direct experimental evidence to support this hypothesis. Moreover, a structure of a complex of BamC and BamD suggests that BamC at least partially occludes this pocket (12). Perhaps BamC is flexible and could regulate access of substrates to the BamD binding site, but this study illustrates the importance of obtaining a structure of the Bam complex—structures of the individual components can be misleading.

BamE consists of two α-helices packed against a three-stranded β-sheet and is structurally homologous to protein inhibitors of β-lactamases. It has also been found that BamE can form dimers and oligomers and may bind phosphatidylglycerol. The functional significance of these observations remains unclear (10, 16, 17). The only clear role for BamE in the Bam complex, which has been established biochemically, is to stabilize the interaction between BamD and BamA (18).

A structure of the Bam complex could help to clarify some of these mechanistic hypotheses. The first structures of the Sec machinery revealed a lateral opening in the

translocase channel that inspired a mechanistic model in which the channel opens like a clamshell to allow the α-helices of inner membrane proteins to insert laterally into the lipid bilayer. The β-barrel of BamA is highly conserved, suggesting that it does not serve simply as a membrane anchor for the POTRA domains and lipoproteins but rather has a functional role. A structure of this part of the protein could be illuminating. It was reported in 2006 that BamA can be over-expressed in high yield as inclusion bodies and folded in vitro to produce a β-barrel, but a structure has not yet been reported (14). The β-barrel of BamA does denature at lower temperature than many other OMPs, and perhaps this instability reflects some flexibility in its structure that has precluded crystallization (19). A complex of BamA and the Bam lipoproteins might be more suitable for crystallization, and the purification of several Bam subcomplexes described in Chapters 2 and 3 made such investigations possible.

4.2 Initial Crystallization Screening of the BamACDE Subcomplex

The four-protein complex lacking BamB was selected for crystallization for several reasons: (1) it can be produced in high yield (~2 mg/L culture); (2) it is stable in several different detergents; (3) it can be produced by in vitro folding of BamA from inclusion bodies, which may limit the lipid contamination of the sample compared to purification directly from outer membranes; and (4) several complexes containing truncated BamA molecules can be purified by the same methods, which might be useful in optimizing any initial crystallization hits. By comparison, the complete five-protein complex must be purified by extraction of BamA and BamB from the outer membrane, which results in lower yields. LPS likely remains associated with the β-barrel of BamA during the purification, and the reconstruction of the complex by mixing membrane extracts of BamAB and BamCDE can result in slightly different ratios of the

different components, leading to inconsistent crystallization results. The five-protein complex is stable in dodecylmaltoside, but some detergents, including octylglucoside, dissociate BamB from BamA. Detergents significantly affect how membrane proteins crystallize, so it is advantageous to be able to purify the complex in a number of different detergents for initial screening.

The BamACDE complex was first purified in octylglucoside (OG), decylmaltoside (DM), and dodecylmaltoside (DDM), and a Phoenix robot was used to screen 960 conditions for crystallization of the complex in each of the different detergents. After three days, small, poorly defined crystals appeared in three conditions at 18 ^{o}C containing the protein complex purified in DDM and the precipitant polyethylene glycol with a molecular weight of 400 (PEG 400) (Table 4.1, entries 1-3). Unfortunately, none of these crystals could be reproduced. However, after one week, crystals appeared in several additional conditions (Table 4.1, entries 4-6). These were all obtained from the protein purified in OG and incubated with the crystallization reagents at 4 ^{o}C. Two of these conditions (entries 4 and 5 in Table 4.1) reproducibly generated crystals and were optimized further.

Table 4.1. Initial BamACDE Crystallization Hits

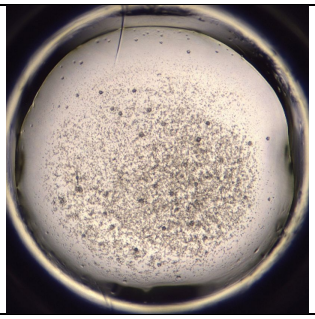
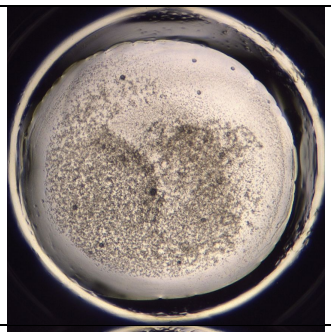
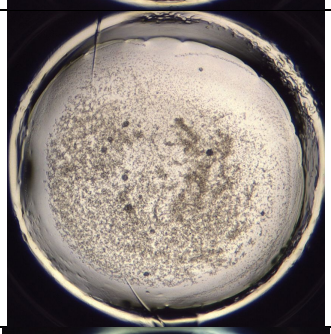
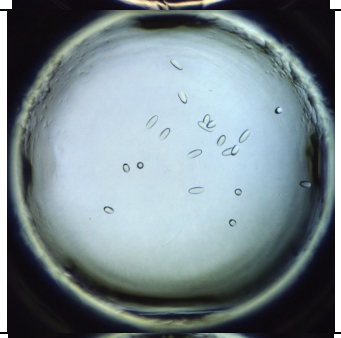
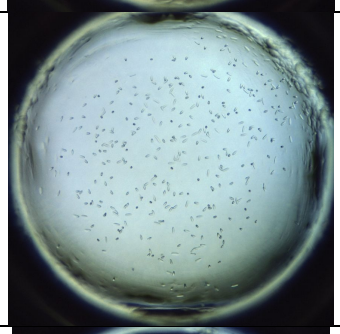
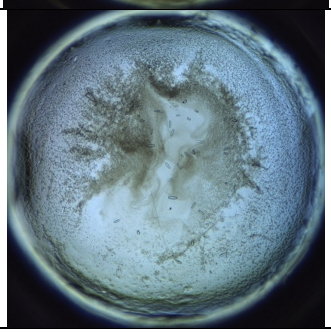
Entry	Purification Detergent	Crystallization Reagent	Crystallization Temperature (^{o}C)	Image of Crystallization Well after Three Weeks
1	0.03% DDM	100 mM trisodium citrate (pH 5.5), 100 mM sodium chloride, 100 mM lithium sulfate, 30% PEG 400	18	

Table 4.1. (Continued)

2	0.03% DDM	100 mM trisodium citrate (pH 5.6), 100 mM sodium chloride, 30% PEG 400	18	
3	0.03% DDM	100 mM trisodium citrate (pH 5.6), 100 mM lithium sulfate, 30% PEG 400	18	
4	1% OG	100 mM trisodium citrate (pH 5.6), 1 M ammonium dihydrogen phosphate	4	
5	1% OG	100 mM sodium acetate (pH 4.6), 400 mM magnesium formate	4	
6	1% OG	100 mM trisodium citrate (pH 5.5), 200 mM sodium acetate, 10% PEG 4000	4	

By screening a range of precipitant concentrations and buffer pH values around these initial hits, crystals of larger size were obtained. Ninety-six different additive compounds were also screened for their effect on crystallization. Small amounts of these compounds were added to the ammonium dihydrogen phosphate crystallization condition (Entry 4 in Table 4.1), and after one week, crystals appeared in a condition containing 50 mM sodium fluoride. Crystals from the ammonium dihydrogen phosphate and magnesium formate precipitant conditions were cryoprotected, mounted, and tested for diffraction at the Advanced Photon Source at Argonne National Laboratory. One of the crystals from the ammonium dihydrogen phosphate condition with added sodium fluoride diffracted weakly to a highest resolution of approximately 11 Å. The diffraction data enabled a tentative assignment of the crystal symmetry to the P622 space group (i.e. a rod with a hexagonal screw axis) and a unit cell size of approximately 114 Å by 114 Å by 751 Å. This space group and unit cell was not observed in any of the published crystal structures, which implies that the crystallized protein complex is a new form. Although the BamA, C, D, and E proteins were purified as a complex, it is possible that they could dissociate or degrade in the crystallization condition and that a subset of them could then crystallize. However, the unit cell of this crystal form is very large, and a comparison of its volume to that expected for a globular protein with a molecular weight of the BamACDE complex (*i.e.* the Matthews coefficient) indicates that it can accommodate all four Bam proteins. The diffraction data obtained from this crystal was, nevertheless, insufficient to determine any structural information.

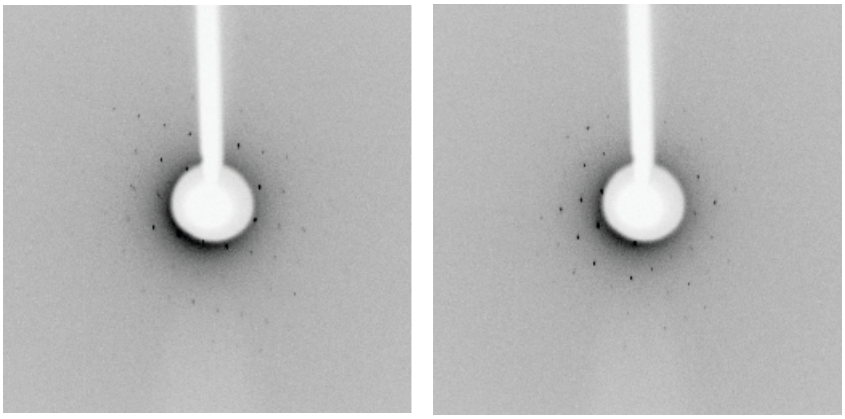


Figure 4.2. Two snapshots of the diffraction produced by the optimized BamACDE crystals. The resolution limits at the edges and corners of the images are 10.5 Å and 7.5 Å, respectively.

To determine whether the small size of these initial crystals resulted in their weak diffraction, larger crystals of the same form were obtained. After approximately two months, crystals that were approximately five times larger (~50 μm by 50 μm by 300 μm) were obtained in conditions containing added ethylene glycol, glycerol, 1,3-propanediol, or acetone. (The BamACDE complex was still purified in OG, and the crystallization solution consisted of 100 mM trisodium citrate (pH 5.6) and 1.1 M ammonium dihydrogen phosphate.) These additive compounds may have improved the solubility of the protein complex in the precipitant solution such that the crystals grew more slowly but to a larger size. These crystals produced slightly stronger diffraction but still with a maximum resolution of approximately 12 Å (Figure 4.2). The space group was again determined to be P622 and the unit cell was 117.19 Å by 117.19 Å by 756.04 Å, but no further information could be obtained. Because the crystals were now larger than the x-ray beam diameter, it was concluded that the low resolution of the diffraction was due to poor packing of the protein in this crystal form.

4.3 Truncated BamACDE Complexes Crystallize in Different Conditions

The POTRA domains of BamA have been shown to be flexible; they crystallized in two different conformations, and NMR and small angle x-ray scattering (SAXS) measurements indicate that they are dynamic with a specific hinge between POTRA domains 2 and 3 (1-3, 20). The truncated BamACDE complexes were therefore screened for crystallization in hopes that removing some of the POTRA domains would improve the protein packing in the crystal and lead to improved diffraction. All of the complexes were purified in OG because this detergent had yielded the most promising crystals in the initial screens, and several complex concentrations were tested. The protein concentration in the crystallization well can alter the rate at which the protein undergoes a phase transition and can thereby affect its ability to crystallize. Complexes lacking one, two, and three POTRA domains crystallized in various conditions (Table 4.2).

Table 4.2. Truncated BamACDE Crystallization Hits

Complex	Entry	Concentration (mg/mL)	Crystallization Reagent	Crystallization Temperature (^{o}C)
BamAΔP1CDE	1	10.5	100 mM HEPES (pH 7.5), 20% Jeffamine M-600	18
	2	10.5	100 mM HEPES (pH 7.5), 100 mM ammonium sulfate, 30% PEG 400	18
	3	10.5	100 mM HEPES (pH 7.5), 100 mM ammonium sulfate, 18% PEG 400	18
	4	10.5	100 mM Tris (pH 8.5), 2.2 M calcium chloride	18
	5	10.5	100 mM Tris (pH 8.5), 30% Jeffamine M-600	18
BamAΔP1-2CDE	6	9.6	100 mM sodium cacodylate (pH 6.5), 150 mM potassium thiocyanate, 20% PEG 550 monomethylether	18
	7	9.6	100 mM Tris (pH 8.5), 2.2 M calcium chloride	18

Table 4.2. (Continued)

BamAΔP1-2CDE	8	9.6	100 mM Bicine (pH 9.0), 100 mM sodium chloride, 30% PEG 550 MME	22
	9	10.2	100 mM HEPES (pH 7.5), 30% PEG 300	22
	10	10.2	100 mM HEPES (pH 7.5), 30% PEG 400	22
	11	10.2	100 mM Tris (pH 8.5), 30% PEG 400	22
	12	10.2	100 mM Tris (pH 8.5), 25% PEG 550 monomethylether	22
BamAΔP1-3CDE	13	16.5	100 mM Tris (pH 8.5), 500 mM potassium dihydrogen phosphate	4
	14	16.5	100 mM trisodium citrate (pH 5.6), 200 mM ammonium acetate, 30% 2-methyl-2,4-pentanediol	4
	15	16.5	100 mM trisodium citrate (pH 5.6), 100 mM magnesium chloride, 4% 2-methyl-2,4-pentanediol	4
	16	7	100 mM sodium acetate (pH 4.5), 200 mM lithium sulfate, 50% PEG 400	4

Many of these conditions produced crystals that were round or oval-shaped. The most promising crystals appeared in condition 6 in Table 4.2; these crystals were hexagonal with sharper edges than many of the other crystal forms (Figure 4.3A). It is unusual for a protein to crystallize in the same form under very different conditions, but the hexagonal shape suggested that the complex may have crystallized in the same P622 space group as was observed previously. We hypothesized that removing the first two POTRA domains eliminated the flexibility at the hinge point between P2 and P3, allowing the complex to form a more ordered crystal. These crystals were optimized by changing the pH and precipitant concentration, by varying the drop size and ratio of precipitant to protein, by screening both traditional small

molecule additives and detergent additives, and by growing new crystals from seeds of older crystals. Larger crystals (~200 μm in each dimension) were obtained by several of these methods. Detergents were also screened as additives to conditions 1 and 8-12 in Table 4.2, and crystals were obtained with several detergents in conditions 1, 8, and 12. Crystals from conditions 1, 6, 8, 10, and 13 (with and without additives) were examined for diffraction at the synchrotron facilities at the Brookhaven and Argonne National Labs. Unfortunately, none of them produced any diffraction. A few crystals from condition 6 were shot at room temperature on the in house x-ray source to determine whether the cryoprotection procedure had damaged the crystals; again, no diffraction was observed.

It is difficult to determine why a protein crystal does not diffract. The fact that crystals form in numerous conditions suggests that these protein complexes can form crystal packing interactions, but there is still a significant amount of disorder in the crystal lattice. Many of the crystals were found to be extremely fragile during the cryoprotection and mounting procedure; this may indicate that they have a high solvent (i.e. water) content and that the protein is not tightly packed. In order to determine whether the disorder could be due to degradation or dissociation of the protein complex during crystallization, crystals obtained in the sodium cacodylate, potassium thiocyanate, and PEG 550 MME condition (condition 6 in Table 4.2) were washed, dissolved, and analyzed on SDS-PAGE (Figure 4.3B).

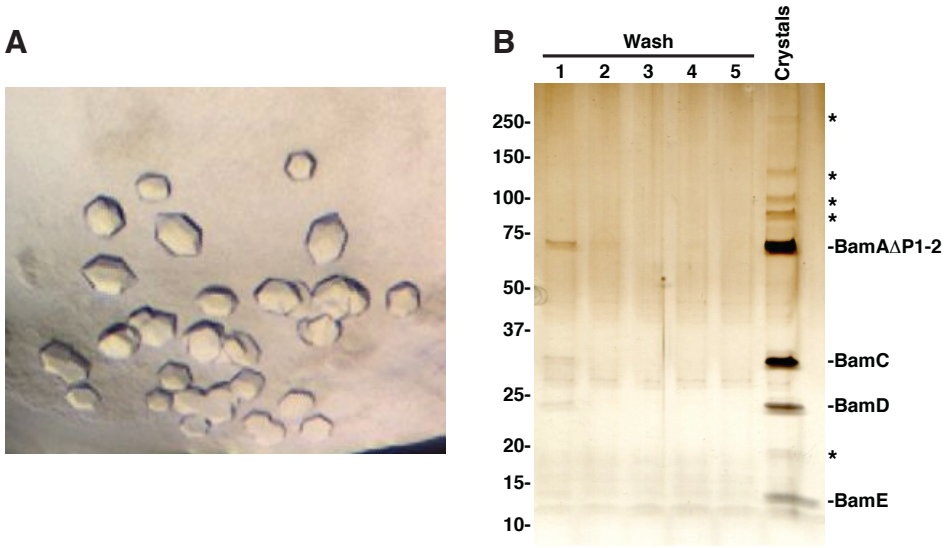
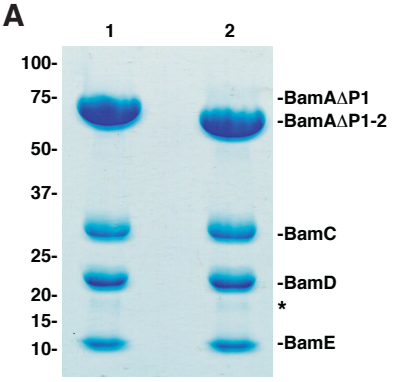


Figure 4.3. Crystals containing the BamAΔP1-2CDE subcomplex. **A.** Crystals with a hexagonal rod-like shape obtained by purifying the subcomplex in octylglucoside and incubating it with sodium cacodylate (pH 5.6), potassium thiocyanate, and PEG 550 monomethylether. **B.** Silver-stained SDS-PAGE of crystals grown in this condition. Crystals were transferred five times to new solutions of the crystallization reagent with added glycerol to separate the crystallized protein from any soluble proteins in the well. The washed crystals were then dissolved in SDS buffer. The crystals contain all four components of the complex and several additional contaminating proteins, which are indicated with stars.

These crystals do contain all four components of the BamAΔP1-2CDE complex, but a few additional bands are also present. The high molecular weight bands are not visible on a coomassie-stained gel of the newly purified complex, and their identities have not yet been determined. It is possible that one of them may contain full-length BamA, which is expressed at native levels in the over-expression strains and therefore could be isolated at low levels with the BamCDE proteins. The band at approximately 20 kDa is present when the BamAΔP1CDE and BamAΔP1-2CDE complexes are newly purified (Figure 4.4A). The proteins in these bands were identified by trypsin digestion and mass spectrometry (MS) at the Taplin Mass Spectrometry Core Facility at Harvard Medical School (Figures 4.4B and C). Most of the peptides obtained from these bands contain sequences from BamC. Peptides from a few other proteins were also identified, but it is likely that BamC is degrading during the purification procedure. The first 78 residues of the BamC sequence were not detected by MS; this part of the protein has been shown

to form an extended loop that binds to BamD (12). The loop was found to be necessary to purify a BamCD complex, but it is possible that BamE could stabilize the BamCD interaction in other ways such that the loop is no longer required. The BamC degradation product might thus co-purify with the subcomplex. A second degradation after lysine 261 would produce a polypeptide with a molecular weight of approximately 20 kDa. The identification of peptides from the C-terminus of the protein may indicate that the degradation is incomplete. The presence of these various degradation products in the crystal lattice would certainly introduce disorder.

A



B

Protein	Number of Peptides	Number of Unique Peptides
BamC	252	27
BamA	40	26
BamD	27	16
50S ribosomal protein L6	17	12
LptE	13	9

C

MAYSVQKSRL AKVAGVSLVL LLAACSSDSR YKRQVSGDEA YLEAAPLAEL
HAPAGMILPV TSGDYAIPVT NGSGAVGKAL DIRPPAQPLA LVSGARTQFT
GDTASLLVEN GRGNTLWPQV VSVLQAKNYT ITQRDDAGQT LTTDWVQWNR
LDEDEQYRGR YQISVKPQGY QQAVTVKLLN LEQAGKPVAD AASMQRYSTE
MMNVISAGLD KSATDAANAA QNRASTTMDV QSAADDTGLP MLVVRGPFNV
VWQRLPAALE KVGMKVTDST RSQGNMAVTY KPLSDSDWQE LGASDPGLAS
GDYKLQVGDL DNRSSLQFID PKGHTLTQSQ NDALVAVFQA AFSK

Figure 4.4. The BamACDE subcomplexes co-purify with a degradation product of BamC. **A.** SDS-PAGE of the purified BamAΔP1CDE (lane 1) and BamAΔP1-2CDE (lane 2) subcomplexes. The bands at approximately 20 kDa were excised from the gel and submitted for mass spectrometry (MS) identification. **B.** The five most abundant proteins in the excised band from the BamAΔP1-2CDE purification. The abundance of peptides from BamC suggests that this contaminant is most likely a degradation product. The MS results from the excised band from the BamAΔP1CDE purification were very similar. **C.** BamC peptide sequences identified by MS. The entire sequence of BamC is illustrated; the portions highlighted in red represent sequences detected by MS.

4.4 Recommendations for Future Crystallographic Efforts

The fact that crystals containing four of the Bam proteins can be generated suggests that it will ultimately be possible to determine a structure of the complex; however, the conditions for crystallization clearly require significant improvement. The homogeneity of the purified complexes can be improved in several ways. First, the degradation of BamC should be addressed either by identifying better purification conditions (e.g. by screening other protease

inhibitors or by eliminating the dialysis step, which significantly lengthens the purification time) or by making conservative mutations in the BamC sequence that would prevent the degradation. It is not ideal to crystallize a mutated protein because it is impossible to know whether the mutation alters the structure of the protein, but the previously determined structure of a BamCD subcomplex indicates that the N-terminal region of BamC forms a long loop, which might tolerate mutations without significant structural changes.

Second, any unfolded BamA in the purified complex should be eliminated. The BamA constructs are expressed as inclusion bodies in the cytoplasm; these are then isolated, dissolved in denaturant, and diluted into detergent to produce the folded β-barrel. The folding is never complete such that some amount of BamA always remains unfolded. The BamCDE lipoproteins are mixed with this material and the four-protein complex is isolated by nickel affinity chromatography in which a histidine tag on BamE is used to pull-down all four proteins. The Bam lipoproteins have higher affinity for folded BamA than for unfolded BamA, so this procedure isolates predominantly the folded form of BamA, but a small amount of unfolded material is also pulled down (Figure 4.5A). Perhaps the POTRA domains of this "unfolded" material are, in fact, structured and thus can bind to the lipoproteins. Preliminary experiments (data not shown) suggest that increasing the amount of BamA mixed with the BamCDE lipoproteins decreases the amount of unfolded, or partially folded, BamA that co-purifies with the folded complex. The folded form can effectively compete for binding to the lipoproteins if a sufficient excess of BamA is provided. Large excesses of BamA should be used in the future to determine whether the "unfolded" BamA can be completely eliminated.

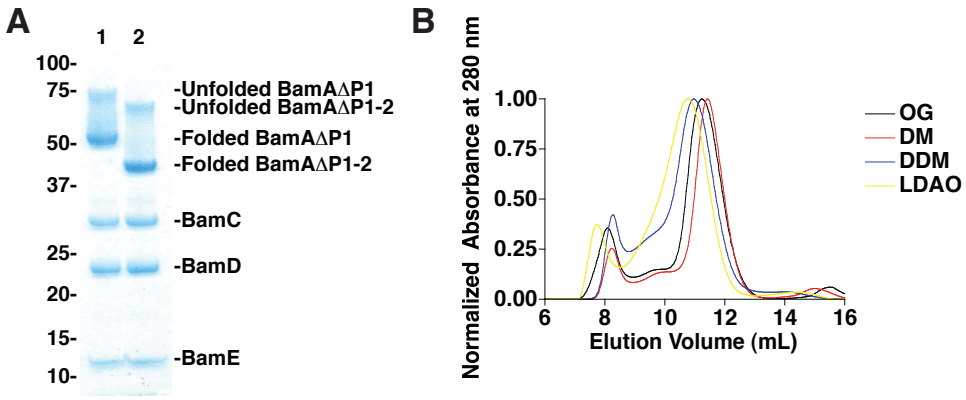


Figure 4.5. The homogeneity of the BamACDE subcomplexes is affected by the purification conditions. **A.** Semi-native SDS-PAGE of the BamAΔP1CDE (lane 1) and BamAΔP1-2CDE (lane 2) subcomplexes indicates that some unfolded BamA co-purifies with these complexes. **B.** Size exclusion chromatograms of the BamACDE complex purified in four different detergents. The complex appears to be more homogeneous in octylglucoside (OG) and decylmaltoside (DM) than in dodecylmaltoside (DDM) or lauryldimethylamine oxide (LDAO).

Third, octylglucoside has proved to be a useful detergent for generating crystals of this complex, but other detergents should also be investigated. Detergents that form smaller micelles, like OG, are thought to be better for crystallography because they may expose more of the soluble regions of the protein, allowing crystal contacts to form more readily (21). The BamACDE complex was also purified in lauryldimethylamine oxide (LDAO) and screened for crystallization, but the complex produced a broader peak on size exclusion chromatography in this detergent, which may indicate increased conformational heterogeneity in the complex, and no crystals formed (Figure 4.5B). Other detergents with more similar properties to OG, including heptylglucoside, nonylglucoside, and octylgalactoside, should be evaluated; they could improve the packing of the complex in the crystal lattice by changing the micelle size without significantly altering the structure of the proteins.

Finally, efforts to identify other Bam subcomplexes that crystallize should continue. Removing some of the lipoproteins or their appended lipids could yield complexes that crystallize in different forms. Limited proteolysis experiments might indicate if there are particularly flexible regions of the Bam proteins that could then be eliminated. Complexes

containing BamB might also exhibit different conformations that would form different crystal contacts. There are a seemingly endless number of possible constructs and conditions to screen, but a structure of the Bam complex will be required to understand its mechanism fully.

4.5 Conclusion

The folding and insertion of a β-barrel into a membrane would seem to require a highly cooperative event. There would be high barrier to inserting individual β-strands into the hydrophobic bilayer because the polar amide bonds of the polypeptide backbone would not be paired in hydrogen bonding interactions. Conversely, assembly of the entire β-barrel prior to insertion would require that the N- and C-termini of the substrate be brought together in the periplasm and then a large number of lipids in the membrane would have to be simultaneously rearranged to allow the folded protein to insert. For these reasons and because β-barrel assembly is not coupled to any external energy source, it is unlikely that the Bam complex sequentially inserts individual secondary structural elements as the Sec machine does or that it drives a prefolded substrate into the membrane as the Get proteins do with tail-anchored membrane proteins (22-25).

It is remarkable that a highly simplified, in vitro system can perform the assembly process at all; the reconstitution is successful in that it recapitulates the cellular process and completes all of the folding and insertion steps. Ironically, a less efficient reconstitution in which the mechanistic steps can be distinguished will be necessary to understand the details how the Bam complex works. The in vitro studies have thus far demonstrated that BamA performs the general steps in the assembly transformation and the lipoproteins adapt that activity to fold the approximately 100 different β-barrels in the *E. coli* OM efficiently. An assay that can

separate the binding, folding, and insertion events is needed to elucidate how BamA interacts with substrates and how the lipoproteins regulate or enhance those interactions. A structure of the Bam complex would also allow us to make rational mutations in the Bam proteins that could be evaluated in vitro. This combination of tools will allow us to dissect this mysterious process and determine whether there are any general principles that guide the assembly of all membrane proteins.

4.6 Materials and Methods

4.6.1 Materials

All cultures were grown in LB media from Difco with the appropriate antibiotics added at concentrations of 50 μg/mL. The n-dodecyl-β-D-maltopyranoside (DDM), n-decyl-β-D-maltopyranoside (DM), n-octyl-β-D-glucopyranoside (OG), and lauryldimethylamine-N-oxide (LDAO) detergents were purchased in >99% purity from Anatrace. The tris(2-carboxyethyl)phosphine (TCEP) was purchased from Hampton Research. The Ni-NTA resin was purchased from Qiagen, and all size exclusion chromatography steps were performed using a Superdex 200 column from GE Healthcare. All protein concentrations were determined using the Bio-Rad DC Protein Assay. Crystal screens were purchased from Hampton Research, Qiagen, and Molecular Dimensions. Buffers, salts, and polyethylene glycol reagents used to reproduce and optimize the initial crystal hits were obtained from Sigma-Aldrich and Hampton Research. The additive and detergent additive screens from Hampton Research were also used to optimize initial crystal hits. Seeding experiments were performed using the Hampton Research Seed Bead Kit according to the manufacturer's instructions. The gel in Figure 4.3B was stained using the Invitrogen SilverQuest Silver Staining Kit.

4.6.2 Expression and Purification of the BamACDE and Truncated BamACDE Subcomplexes

As described in Chapter 3, the BamA constructs were expressed without their signal sequences as inclusion bodies in BL21(DE3) cells carrying pCH103, pSK131, pCH36, and pSK133 (for full-length, ΔP1, ΔP1-2, and ΔP1-3 BamA, respectively). Cultures (1.5 L) of these strains were grown to OD_{600} = 0.4-0.6 at 37 ^{o}C, and expression of the proteins was induced with

0.1 mM IPTG for 3-4 hours. The cells were harvested, resuspended in TBS (pH 8) (*i.e.* 20 mM Tris-HCl (pH 8), 150 mM NaCl) with 0.1 mg/mL deoxyribonuclease, 0.1 mg/mL ribonuclease, and 0.1 mg/mL lysozyme, and lysed by French press. They were then centrifuged at 5,000 x g for 10 min at 4 ^{o}C. The pelleted inclusion bodies were resuspended in 10 mL of TBS (pH 8), centrifuged again at 5,000 x g for 10 min at 4 ^{o}C, and finally dissolved in 10 mL of 8 M urea. These urea solutions were incubated on a rocker at room temperature for approximately one hour and then centrifuged at 18,000 x g for 10 min at 4 ^{o}C to remove any undissolved material. The supernatants was then diluted ten-fold by dropwise addition to stirred solutions of TBS (pH 8), 0.5% LDAO. They were incubated overnight at 4 ^{o}C with stirring to allow the β-barrels of the BamA constructs to fold.

The BamCDE proteins were expressed in BL21(DE3) cells carrying pSK46 and pBamE-His as described in Chapter 2 in the reconstruction of the five-protein complex. The cells from 3 L of these cultures were lysed, and their membranes were isolated, solubilized, and dialyzed overnight as described previously. These solubilized membrane solutions were added to the BamA solutions in TBS, 0.5% LDAO and stirred for approximately 15 minutes. The BamACDE subcomplexes were then isolated by Ni-NTA affinity chromatography. The Ni resin was washed with a solution of TBS (pH 8), 40 mM imidazole, and the chosen detergent (0.05% DDM, 0.2% DM, 1% OG, or 0.05% LDAO), and the proteins were eluted with a solution of TBS (pH 8), 200 mM imidazole, and the same detergent. These eluates were concentrated and subjected to size exclusion chromatography in TBS (pH 8), 1 mM TCEP, and the appropriate detergent (0.03% DDM, 0.2% DM, 1% OG, or 0.05% LDAO). The central fractions from the major peak in each of the size exclusion chromatograms were collected and concentrated.

4.6.3 Initial Crystallization Screening

The BamACDE subcomplexes were screened in numerous conditions using commercial screens designed for crystallization of soluble and membrane proteins. 96-well screening plates were set-up using an Art Robbins Instruments Phoenix robot in the MIT Biology Department with a drop size of 300 nL (150 nL of protein solution and 150 nL of the crystallization reagent) and a reservoir volume of 70 μL. The screening plates were incubated in Formulatrix Rock Imagers at 4 ^{o}C or 18 ^{o}C and observed using the Rockmaker software.

4.6.4 Secondary Screening and Optimization

Conditions that produced crystals in the nanoliter-sized drops were reproduced at larger scale in 24-well plates with a typical drop size of 1 μL (0.5 μL of protein solution and 0.5 μL of the crystallization reagent) and a reservoir volume of 700 μL. Typically a range of pH values and precipitant concentrations around the initial crystallization condition were tested. Crystals that could be reproduced at this scale were then screened with traditional small molecule and detergent additives to determine whether any of these would affect the rate of crystallization and thereby lead to different diffraction. Crystal seeding was employed to obtain larger crystals in a few cases. Preformed crystals were fragmented using the Hampton Research Seed Bead Kit and then added to freshly purified protein in the same crystallization reagent; typically lower concentrations of the precipitant were used in these experiments to decrease the spontaneous nucleation of new crystals.

4.6.5 X-ray Diffraction and Analysis

Crystals were examined for diffraction at 100 K on the 24-ID-E beamline at the Advanced Photon Source (APS) at the Argonne National Labs and on the x25 beamline at the National Synchrotron Light Source (NSLS) at the Brookhaven National Labs. The diffraction images shown in Figure 4.2 were collected at APS with a 70 μm beam at 35% transmission. The data were indexed in iMosflm and the Matthews coefficient was calculated in the Matthews program.

4.6.6 SDS-PAGE Analysis of Crystallized Proteins

Three crystals of BamAΔP1-2CDE grown in 100 mM sodium cacodylate (pH 6), 150 mM potassium thiocyanate, and 13% PEG 550 MME were transferred from the crystallization solution to 1 μL of the reservoir solution with added (15%) glycerol using a mounted cryoloop. The crystals were transferred five more times to new 1 μL drops of the same solution. The first five empty drops and the final drop containing the crystals were mixed with 2% SDS sample buffer, boiled for ten minutes, and run on SDS-PAGE at 200 V for 45 minutes. The gel was then silver stained to detect the proteins in the wash solutions and in the dissolved crystals.

4.6.7 Mass Spectrometry Identification of Protein Contaminants in the Purified BamACDE Subcomplexes

Newly purified BamAΔP1CDE and BamAΔP1-2CDE complexes were run on SDS-PAGE at 200 V for 45 minutes. The gel was stained with coomassie blue and the bands of unknown identity at ~20 kDa were excised. These were submitted to the Taplin Mass Spectrometry Core Facility at Harvard Medical School where they were subjected to in-gel

trypsin digestion. The sequences of the tryptic peptides were determined by liquid chromatography-tandem mass spectrometry (LC/MS/MS) and compared to the *E. coli* protein database to identify the proteins from which the peptides originate.

4.7 References

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